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Mechanistic bases for differences in passive absorption

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Summary

Increasing evidence indicates that small birds have more extensive non-mediated, paracellular intestinal absorption of hydrosoluble compounds than do mammals, although studies have not employed uniform methodologies or demonstrated differences at the tissue level. The mechanistic bases behind apparent species differences are poorly understood. We show using uniform methodology at the whole-animal level that intact, unanesthetized pigeons had significantly higher absorption of L-arabinose and L-rhamnose, two water-soluble compounds used to measure paracellular absorption, than similarly sized laboratory rats. The species differences were also evident using perfused isolated loops of duodenum, showing that the difference in paracellular absorption occurred at the tissue level, even when D-glucose absorption rates (transcellular+paracellular) were similar between the two species. The greater absorption of these probes in pigeons could not be explained by mediated uptake of the putative paracellular probes, or by increased nominal surface area, increased villus area or increased number of tight junctions. Rats and pigeons had comparable absorption of larger probes, which is consistent with similar effective pore size of the tight junction between enterocytes. The elimination of these mechanistic explanations might suggest that pigeon intestine has relatively higher paracellular solvent drag, but pigeon duodenal segments did not have higher net water absorption than rat duodenal segments. Whatever the exact mechanism(s), the paracellular pathway of both species limits substantial (>5%) fractional absorption to molecules smaller than about 4.8 Å (M_r ca. 350), and permeability to smaller molecules at the tissue level is higher in pigeons than in rats.

Key words: paracellular nutrient absorption, tight junction, solvent drag, gut morphology.

Introduction

The passive diffusion of molecules across the phospholipid bilayer membrane of enterocytes is correlated with their lipid–water partition coefficient (Diamond and Wright, 1969). Water-soluble molecules, like glucose, are not permeable in the lipid bilayer, thus this membrane is a limiting factor for the absorption of hydrophilic molecules. Unless they are taken up by specialized transporters and moved transcellularly, medium to large hydrophilic molecules presumably permeate across the small intestinal mucosal epithelium primarily through the paracellular pathway (Powell, 1987).

Notwithstanding the qualitative similarities between mammalian and avian species in paracellular absorption, researchers have sometimes noted differences in the magnitude of paracellular absorption among mammals (Bijlsma et al., 1995; Delahunty and Hollander, 1987; He et al., 1998; Pappenheimer, 1990) or between mammals and birds (Chediack et al., 2003). Concerning the latter comparison, although many of the studies on birds provide evidence of much higher paracellular absorption (Afik et al., 1997; Caviedes-Vidal and Karasov, 1996; Karasov and Cork, 1994) than observed in mammalian species (cf. Bijlsma et al., 1995; Delahunty and Hollander, 1987; Pappenheimer, 1990), studies have not employed uniform methodologies, and the mechanistic bases behind apparent species differences are poorly understood. Also, if body size influences reliance on paracellular absorption (Pappenheimer, 1998), then this factor should be taken into account. Here, in a series of experiments with similarly sized (300–500 g) pigeons and laboratory rats, we find apparently higher paracellular absorption in pigeons, and test a number of mechanistic bases for this difference.

We used standard methods from pharmacokinetics to measure the whole-organism fractional absorption of L-arabinose (M_r=150.1) and L-rhamnose (M_r=164.2), non-metabolized hydrophilic carbohydrate probes that lack affinity for intestinal mediated uptake mechanisms. These probes are commonly used in tests of passive (non-carrier-mediated) intestinal permeability (reviewed in Travis and Menzies, 1992). Based on previous studies in mammals and small birds (see above), we hypothesized that pigeons would absorb both probes to a greater extent than rats, and we predicted that both species would absorb L-arabinose (smaller molecular size) more than L-rhamnose, consistent with the sieving characteristic of the tight junction (reviewed by Chediack et al., 2003).

To ensure that higher absorption in the pigeon was not due to mediated uptake of presumed paracellular probes, we tested

whether uptake of tracer amounts of the probes by everted intestinal sleeves *in vitro* were inhibited by high concentrations of either the carbohydrate probes themselves or D-glucose. Because greater absorption can occur simply due to longer contact time with the gut epithelium (Lennernas, 1995) as well as differential gastric evacuation rates, we used isolated perfused intestinal loops to test whether differences in absorption of these same probes are maintained at the level of the tissue perfused at the same rate. We also predicted that clearance of L-arabinose from perfusion solutions would be greater than L-rhamnose in both species, comparable to our predictions in whole animal experiments.

Pappenheimer and Reiss's revised version of the Kedem–Katchalsky equation (Kedem and Katchalsky, 1958; Pappenheimer and Reiss, 1987) of clearance of a probe through porous epithelia predicts that a greater solvent flow rate and/or a larger tight junction effective pore size may explain higher clearance in pigeons *vs* rats. Tight junctional proteins (Schneeberger and Lynch, 2004) that form a sieve with larger effective apertures would allow greater clearance, and thus more extensive paracellular absorption, of both small and large probes. The clearance of carbohydrate probes in rats becomes very low for molecules larger than the disaccharide lactulose, of M_r=342 and molecular radius ca. 0.49 nm (Hamilton et al., 1987). Therefore, also using isolated perfused loops of intestine, we compared both pigeons and rats for clearance of large molecular mass probes (cellobiose: M_r=342.3; raffinose: M_r=594.5). If pigeons have more extensive paracellular transport because of increased solvent flow, then one might expect greater net water flux under physiological conditions (isosmotic perfusion solutions). Consequently, we also measured net water flux under conditions in which carbohydrate probe absorption was higher in pigeons than in rats, also using isolated intestinal loops.

Because higher absorption at the level of either the whole animal or per unit length of intestine could occur due to increased nominal surface area (the area of a smooth bore tube), increased mucosal surface area (due to magnification by villi), or increased number of tight junctions, we compared intestinal morphometric measures in the two species.

Material and methods

Animals and their maintenance

Male Sprague-Dawley rats (*Rattus norvegicus* L.; 325–500 g) were purchased from Harlan World Headquarters (Indianapolis, IN, USA). Rats had access *ad libitum* to water and rat chow (Harlan Teklad, Madison WI, USA). The rats were housed in pairs in shoebox cages under relatively constant environmental conditions (22±1°C, relative humidity 35±3%) and a reverse lighting schedule of 12 h:12 h light:dark (20:00 h–08:00 h light; 08:00 h–20:00 h dark). Pigeons (*Columba livia* L.; ~300 g) were purchased from a Wisconsin breeder (Earl Ditsch Farm, Oregon, WI, USA). Pigeons had access to *ad libitum* water and pelleted pigeon feed (Purina Pigeon Checkers, Purina Mills, Inc., Richmond, IN, USA). Pigeons were housed singly in cages under relatively constant environmental conditions (20.9±0.1°C, relative humidity of 48.4±0.1%) on a diurnal light schedule of 15:20 h:8:40 h light:dark (05:20 h–20:40 h light; 20:40 h–05:20 h dark). All animals were acclimated to the laboratory for at least a week before they were used in experiments. The University of Wisconsin College of Agricultural and Life Sciences Animal Care and Use Committee approved all animal care and experimental procedures for this study.

Test compounds

Carbohydrates were purchased from Sigma Chemicals (St Louis, MO, USA): L-arabinose (M_r=150.1), L-rhamnose (M_r=164.2), mannitol (M_r=182.2), D-glucose (M_r=180.2), L-glucose (M_r=180.2), 3-*O*-methyL-D-glucose (M_r=194.2), lactulose (M_r=342.3), cellobiose (M_r=342.3), raffinose (M_r=594.5) and stachyose (M_r=666.6). Radiolabeled chemicals were purchased from American Radiolabeled Chemicals Inc. (ARC, St Louis, MO, USA) and NEN (PerkinElmer Life Sciences, Boston, MA, USA).

Fractional absorption of probes measured in vivo

The night before a trial (during the animals' normal inactive period), food was withheld. Prior to administration of the gavage solution containing test molecules, rats and pigeons were gavaged twice (2.5 ml each for rats, 3.0 ml each for pigeons) with an isosmotic solution (rats: 290 mmol kg^{-1}, pigeons: 350 mmol kg^{-1}) containing 30 mmol l^{-1} NaCl, 10 mmol l^{-1} D-glucose and mannitol [either 280 mmol l^{-1} for pigeons or 230 mmol l^{-1} for rats, to be isosmotic with avian and mammalian plasma, respectively (Charkoudian et al., 2005; Goldstein and Zahedi, 1990)]. Animals were then gavaged with an isosmotic solution containing 30 mmol l^{-1} NaCl, 40 mmol l^{-1} 3-*O*-methyl-D-glucose, 40 mmol l^{-1} L-arabinose and 40 mmol l^{-1} L-rhamnose at a dose of 1.0% body mass. Other inert probes (mannitol, lactulose, raffinose and stachyose) and NaCl were included in the solution to balance osmolality. Inclusion of Na^+ also provides an essential ion for Na^+-coupled D-glucose absorption, although it is not strictly necessary in this kind of whole-animal study because animals would still absorb nearly all glucose even if the diet is low in Na^+; this is because additional Na^+ is secreted into the intestinal lumen together with bicarbonate and diffuses from blood (Brody, 1999). In a separate experimental trial, rats and pigeons were injected (0.6% body mass) with 2.5 mmol l^{-1} NaCl, 50 mmol l^{-1} 3-*O*-methyl-D-glucose, 50 mmol l^{-1} L-arabinose and 50 mmol l^{-1} L-rhamnose. The injection site was the pectoralis muscle in pigeons and the peritoneal cavity in rats. Blood samples were collected at t=0 (background), 5, 10, 15, 20, 30, 45, 60, 90, 150 and 240 min post-injection or -gavage. Syringes were weighed before and after dosing animals to determine the actual dose administered. Osmotic pressures of solutions were measured (Wescor VAPRO 5520, Logan, UT, USA) prior to administration and averaged 320±7 mmol kg^{-1} for rats and 365±3 mmol kg^{-1} for pigeons to be isosmotic with mammalian and avian plasma, respectively (Charkoudian et al., 2005; Goldstein and Zahedi, 1990).

Pharmacokinetic calculations of absorption

The plasma concentrations C (ng probe mg^{-1} plasma) were plotted as a function of sample time t (min). The probe amounts absorbed were calculated from the areas under the post-gavage

and post-injection plasma curves (AUC=area under the curve of plasma probe concentration *vs* time). Fractional absorption (F) was calculated as F=(AUC by gavage/dose$_{gavage}$)/(AUC by injection/dose$_{injection}$) (Ritschel, 2004). This method of calculating F relies on no major assumptions about compartments or kinetics. Using typical pharmacokinetic procedures (Ritschel, 2004) the AUC from t=0 to t=x min (time of final blood sampling) was calculated using the trapezoidal rule. The AUC from t=x min to t=∞ was calculated by dividing C_x (plasma concentration at time of final blood sampling) by K_{el}. K_{el} is the rate constant describing the probe loss from the systemic circulation by elimination. This parameter was estimated by regressing the last two log-transformed plasma concentrations C (ng probe mg^{-1} plasma) against t (min) and calculating the slope. The time course was analyzed assuming first order elimination. The peak concentration post-injection reflects the effective pool size and the rate of distribution relative to the rate of elimination. The injection data could be used to calculate the effective pool size and rate of elimination, which indeed may differ between the species. The AUC method to measure fractional absorption, however, accounts for such differences.

Everted sleeve uptake experiments

We measured uptake of [^{14}C]L-arabinose, [^{3}H]L-rhamnose, [^{3}H]lactulose, [^{3}H]- or [^{14}C]L-glucose and [^{14}C]D-glucose into the tissue across the brush-border membrane as described elsewhere (Chang et al., 2004; Karasov and Diamond, 1983) and as previously applied in laboratory rats (Green et al., 2005; Karasov and Debnam, 1987) and pigeons (Obst and Diamond, 1989). The first four radiolabeled solutes are thought to be absorbed passively *via* the paracellular pathway, and thus their uptake should not be inhibitable by either themselves or by unlabeled D-glucose, whereas radiolabeled D-glucose uptake should be inhibitable by unlabeled D-glucose (our positive control). Thus, our goal in these experiments was not to measure passive absorption in this preparation of isolated tissue (it is not suited for this measurement), but to confirm that the first four solutes are not absorbed by an active or mediated process. We routinely inspected adjacent tissue sections histologically for signs of villus damage (Green et al., 2005; Starck et al., 2000). Briefly, 1 cm sleeves of everted tissue were preincubated for 5 min in 40°C Ringer's solution and suspended for 4 min (in the case of D-glucose uptake, 2 min) over a stir bar spinning rapidly in a solution containing the labeled probe and either 100 mmol l^{-1} mannitol (the control), 100 mmol l^{-1} D-glucose or 100 mmol l^{-1} of the respective unlabeled probe. Incubation solutions also contained (in mmol l^{-1}) 100 NaCl, 4.7 KCL, 2.5 $CaCl_2$, 1.2 KH_2PO_4, 1.2 $MgSO_4$, and 20 $NaHCO_3$ and made isosmotic (rats: 290 mmol kg^{-1}; pigeons: 350 mmol kg^{-1}) with mannitol, and were oxygenated with 95% O_2 and 5% CO_2. After incubation, issues were blotted, removed from the rod, weighed, incubated in tissue solubilizer (Soluene-350, Packard, Meriden, CT, USA), and counted in a scintillation cocktail (ICN Ecolume with 5% acetic acid; Irvine, CA, USA). To correct for non-absorbed solute in adherent mucosal fluid, we used tracer concentrations of membrane-impermeable marker {1,2-[^{3}H]polyethylene glycol (PEG), M_r=4000}.

Recirculating intestinal perfusions

Animals were anesthetized with isoflorane (2–4.5%) and oxygen (1–2 l min^{-1}) delivered by an anesthesia machine (Surgivet Anesco Isotec 4, Waukesha, WI, USA) and maintained at a constant body temperature of 37–38°C using an electric heating pad and a Deltaphase isothermal pad (Braintree Scientific, Inc., Braintree MA, USA). A peritoneal incision was made and a 5 cm segment (pigeons) or an 18 cm segment (rats) of intestine distal (1–2 cm) to the stomach was identified and cannulated at proximal and distal ends with flexible plastic tubing. We perfused the proximal region of the small intestine because isolating more distal gut sections in pigeons would require additional invasive procedures that would risk puncturing air sacs. A solution of 0.9% NaCl was flushed through the segment to remove digesta. A prewarmed Ringer's solution (included 70 mmol l^{-1} Na^+) containing 50 mmol l^{-1} D-glucose, tracer amounts of [^{14}C]D-glucose and 1.5 mmol l^{-1} each of L-arabinose, L-rhamnose, cellobiose and raffinose, was constantly stirred and recirculated using a Manostat Carter 8/3 cassette pump (Barnant Company, Barrington, IL, USA) through the intestinal loop for 3 h at 1 ml min^{-1} [methods based on Ma et al. (Ma et al., 1991)]. We included 50 mmol l^{-1} D-glucose in the perfusion solution in order to more saturate Na^+-D-glucose transporters and maximize paracellular absorption (Pappenheimer, 1993; Pappenheimer and Reiss, 1987). Osmotic pressures of solutions were measured (Wescor VAPRO 5520) prior to administration and isosmotic solutions averaged 290±2 and 354±1 mmol kg^{-1} for rats and pigeons, respectively. The initial perfusion volume was 30–40 ml and aliquots of 0.7 ml were taken at time=0 and every 30 min and weighed. The incision site was covered with moistened gauze and plastic wrap and was periodically moistened with warmed saline solution (0.9%). The perfusate was weighed pre- and post-perfusion. Air was pumped through the gut segment after the perfusion was complete until all fluid returned to the perfusion reservoir (enclosed to eliminate evaporative loss). The animal was euthanized with CO_2, and the length of the perfused segment was measured. After 2 h (when probe clearance was calculated), there was no significant difference between final paracellular probe concentrations between species ($F_{1,31}$=0.28; P=0.60). Mean final concentrations for L-arabinose, L-rhamnose, cellobiose and raffinose (in mmol l^{-1}) were 1.1±0.2, 1.3±0.1, 1.4±0.04 and 1.6±0.2 for pigeons, and 0.8±0.1, 1.4±0.1, 1.4±0.04 and 1.6±0.1 for rats. Pigeons had a significantly greater final concentration of D-glucose than rats (in mmol l^{-1}: pigeons: 44.9±1.9; rats: 27.3±2.7; t_5=11.9; P<0.001). We did not check villus morphology in segments from perfused animals, but the measured rates of D-glucose absorption did not significantly decline over the course of our experiment (first hour *vs* second hour within animals: t_{10}=–2.0; P=0.07).

Radionuclide activity in samples was measured by scintillation counting (Wallac Winspectral 1414, PerkinElmer Life and Analytical Sciences, Inc, Wellesley, MA, USA, using ICN Ecolume scintillation fluid, ICN, Irvine CA, USA) and concentration of inert probes was measured using High Performance Liquid Chromatography (HPLC; see below). Net water flux was determined by subtracting the perfusate mass post-perfusion from mass pre-perfusion and was normalized to length of intestine loop and total time of perfusion [net water

flux values are reported as μl min^{-1} cm^{-1} (see Krugliak et al., 1989; Ma et al., 1991)]. Negative values of net water flux indicate net water secretion, and positive values indicate net water absorption. The perfusion reservoir was sealed to eliminate evaporative loss, and no radioactivity was detected in the peritoneal cavity of animals after perfusions (indicating no leakage of perfusate). Carbohydrate probe absorption was measured as loss from the perfusate min^{-1} perfusion cm^{-1} intestine, according to the following equation:

$$\text{Probe absorbed (nmol min}^{-1}\text{ cm}^{-1}) = ([\text{Probe}]_{\text{initial}} \times \text{Perfusate mass}_{\text{initial}}) - ([\text{Probe}]_{\text{final}} \times \text{Perfusate mass}_{\text{final}}) / (\text{Perfusion time} \times \text{Segment length}) .$$

Because absorption of these probes is presumably passive (tested in this study), then the rate of absorption (nmol min^{-1} cm^{-1}) is directly related to the probe concentration. In order to correct for the small differences in concentration of probes over the course of the experiment, the rate of absorption results for paracellular probes are expressed as probe clearance (μl min^{-1} cm^{-1}), which was calculated by dividing the value from the above equation by $[(C_{\text{initial}}-C_{\text{final}})/\ln(C_{\text{initial}}/C_{\text{final}})]$, where C is concentration (Fine et al., 1993; Sadowski and Meddings, 1993). D-glucose absorption is expressed as a rate of absorption (nmol min^{-1} cm^{-1}) because it is absorbed by both active and passive processes.

Morphological measurements

Animals were euthanized with CO_2, and their gastrointestinal tract (distal to stomach) was removed. Total length of small intestine (cm) was measured, as was the circumference (cm; 1–2 tissue sub-sections were used for circumference measurements in each third of intestine). Nominal surface area (cm^2) was calculated as the sum of the products of the lengths of the thirds multiplied by the average circumference for the third of the intestine. Adjacent segments to those segments used for measurement of circumference were fixed in 10% formalin, and 5 μm sections were cut on glass slides and stained with Haematoxylin and Eosin (H&E). Measurements of villus amplification ratio (increased area due to villi) were based on the calculation in Kisielinski et al. (Kisielinski et al., 2002). Briefly, the villus length, villus width and crypt width of 25 villi per animal were measured at 40× total magnification using ImageJ software (Abramoff et al., 2004).

The number of enterocytes per unit length villus (10 villi per animal) was counted using light microscopy at 400× total magnification. The density was multiplied by the average area of a villus, the nominal surface area, and the villus amplification ratio to yield the total number of intestinal enterocytes in the small intestine.

Analyses

Plasma and perfusion sample analysis

Blood samples were centrifuged and plasma samples were loaded into preweighed 1.5 ml microcentrifuge tubes equipped with 30K Nanosep filters (Pall Corporation East Hills, NY, USA). Plasma was initially filtered with 50 μl dH_2O (14 000 ***g*** for 30 min), followed by a rinse step with an additional 100 μl dH_2O (14 000 ***g*** for 140 min) to ensure high carbohydrate probe recovery. Plasma samples were subsequently dried at 65°C and stored frozen at –80°C until analysis.

Carbohydrate probes (L-arabinose, L-rhamnose and cellobiose) in plasma and perfusion samples were derivatized for HPLC fluorescence detection by reductive amination with anthranilic acid (2-aminobenzoic acid), following published procedures (Anumula, 1994; Du and Anumula, 1998), with minor modifications. Briefly, dried plasma samples were reconstituted with 50 μl dH_2O or an aliquot of 50 μl of perfusion solution was removed and mixed with 50 μl of anthranilic acid reagent solution. The anthranilic acid reagent consisted of 30 mg ml^{-1} anthranilic acid and 20 mg ml^{-1} sodium cyanoborohydride dissolved in a previously prepared solution of 5% sodium acetate•$3H_2O$ and 2% boric acid in methanol. Samples were transferred to a screw-cap glass autosampler vial and heated at 65°C for 3 h. After cooling to ambient temperature, 300 μl of HPLC solvent A (see below) was added to vials, which were mixed vigorously in order to expel the hydrogen gas evolved during the derivatization reaction.

The carbohydrates in derivitized plasma samples were separated by HPLC (Beckman-Coulter 508 Autosampler, System Gold 126 Solvent Module, 32 Karat Software, v. 5.0, Build 1021, Beckman-Coulter, Fullerton, CA, USA). 20 μl of derivitized plasma samples was injected on a C-18 reversed phase column (Water Pico Tag; 150×3.9 mm; Waters Corporation, Milford, MA, USA) maintained at 23°C (Alltech 530 column heater, Alltech Associates, Inc., Deerfield, IL, USA) using a 1-butylamine-phosphoric acid-tetrahydrofuran mobile phase system at a flow rate of 1 ml min^{-1}. Solvent A consisted of 0.2% 1-butylamine, 0.5% phosphoric acid, and 1% tetrahydrofuran (inhibited) in HPLC grade water [18.2 MΩ resistance produced in-house, further filtered through a 0.45 μm hydrophilic polypropylene membrane filter (GH Polypro, Pall Gelman Sciences, Ann Arbor, MI, USA), or purchased] and solvent B consisted of equal parts solvent A and HPLC grade acetonitrile. Table 1 describes the gradient elution program used for the separation. Carbohydrate probes in plasma samples were quantified by a fluorescence spectrophotometer with the following settings: excitation wavelength 230 nm, slit width 10 nm; emission wavelength 425 nm, slit width 5 nm; sensitivity=1; 'normal' setting for lamp mode, photomultiplier gain and response time [(Anumula, 1994); Perkin-Elmer 650-LC, PerkinElmer Life Sciences, Inc., Boston, MA, USA].

Table 1. *HPLC gradient program for carbohydrate derivative separation*

Time (min)	Solvent A (%)	Solvent B (%)
0 (initial conditions)	90	10
5.5	90	10
6	80	20
12	66	34
13 (wash)	0	100
16	0	100
18	90	10
23	90	10

Changes in solvent composition over time are linear. See Materials and methods for details of solvent composition.

Aliquots of perfusion samples were filtered using Pall Life Sciences Acrodisc LC 13 mm diameter syringe filters with 0.2 μm PVDF membranes (VWR International, Buffalo Grove, IL, USA). 10 μl of filtered perfusion samples was injected on a Prevail Carbohydrate ES column maintained at 23°C (Alltech 530 column heater, Alltech Associates, Inc., Deerfield, IL, USA). Cellobiose and raffinose were quantified by an Evaporative Light-Scattering Detector (Alltech ELSD 500). The mobile phase was 70% acetonitrile and 30% HPLC grade water (see above). Samples had an HPLC time program with a 0.5 min gradient down to 60% acetonitrile and 40% water and a 5 min equilibration back to the initial ratio of solvents. Clearance of cellobiose from the perfusion solution was the same regardless of detector (ELSD=1.82±1.38 μl min^{-1} cm^{-1}; fluorescence spectrophotometer=2.81±0.43 μl min^{-1} cm^{-1}; t_{10}=0.81; P= 0.44). Clearance of cellobiose detected *via* the fluorescence spectrophotometer is reported in the Results.

Statistical analysis

Results are expressed as means ± s.e.m. Estimates of F were arcsin-square-root transformed prior to statistical analyses. F estimates greater than 1 were set to equal 1 (applicable only to 3-*O*-methyl-D-glucose). For some analyses, F and clearance of probes were normalized to the square root of the molecular mass of the probe to correct for differences in diffusivity (Smulders and Wright, 1971). For tissues incubated in various solutions, uptake values (μl mg^{-1} min^{-1}) were normalized to those for adjacent tissues incubated in mannitol (the control) and one sample one-tailed t-tests were conducted to determine if the normalized uptake was significantly different from 1. Repeated-measures analysis of variance (R-M ANOVA: Systat Version 10, Systat Software Inc., Point Richmond, CA, USA) was used to test for differences in probe absorption and uptake, as well as net water flux between rats and pigeons, and to test for the effect of probe size on absorption.

Results

In vivo probe fractional absorption

Carbohydrate probes fed by gavage appeared in the plasma by 5 min post-gavage (first blood sampling time). In pigeons, L-arabinose and L-rhamnose plasma concentrations peaked at 90 min and 3-*O*-methyl-D-glucose peaked at 45 min post-gavage. In rats, L-arabinose peaked at 30 min, L-rhamnose peaked at 90 min and 3-*O*-methyl-D-glucose peaked at 20 min post-gavage. After injection, probes were cleared rapidly with ⩾90% of elimination occurring over the course of the 4 h experiment (Fig. 1). Fitting the elimination data to a mono-exponential elimination model gave values of r^2>0.99 for L-rhamnose, L-arabinose and 3-*O*-methyl-D-glucose in both rats and pigeons, which supports our method of estimating the residual AUC past 240 min using the apparent elimination rate constants (K_{el}; see Materials and methods). The elimination rates in the injection trials did not differ significantly from those in the gavage trials

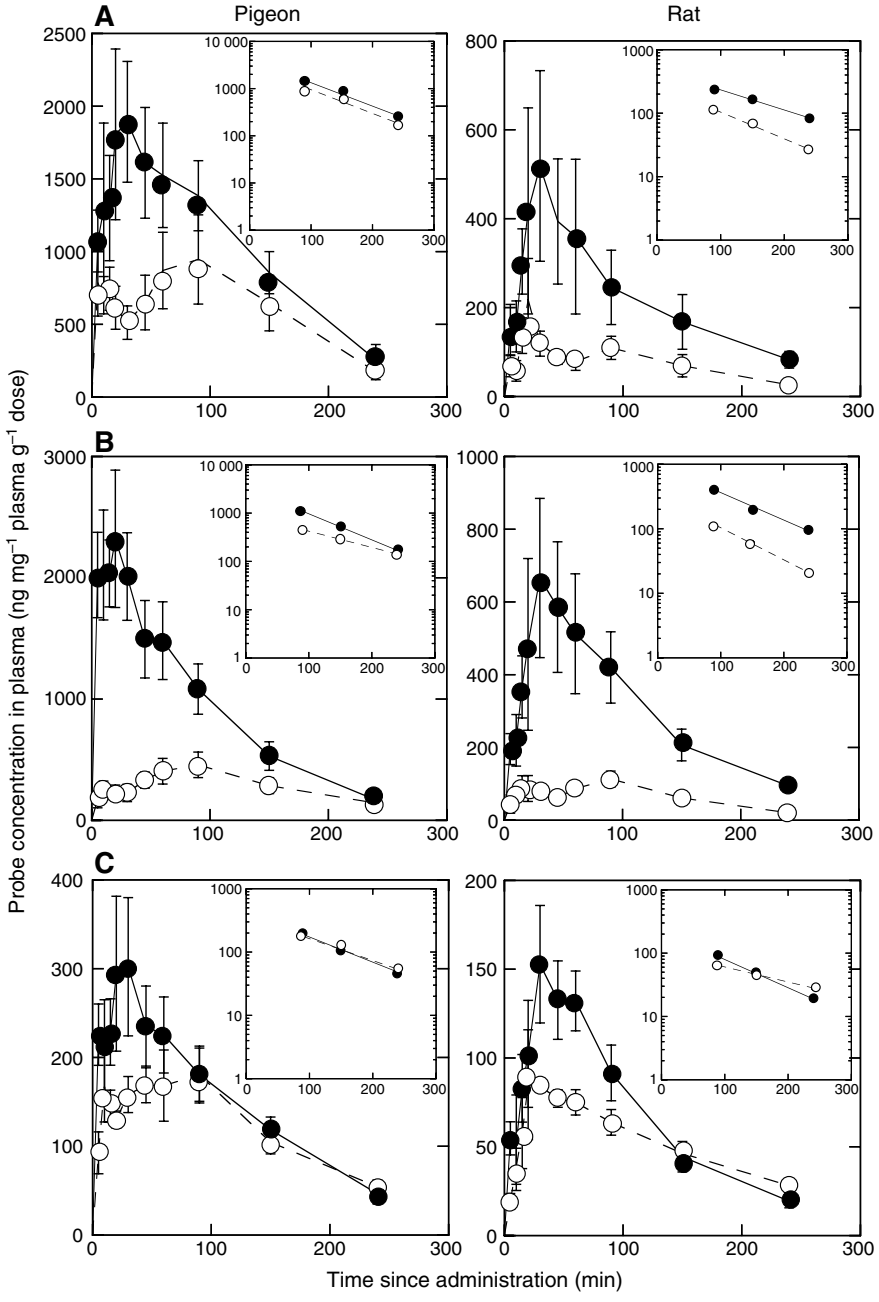

Fig. 1. (A) L-arabinose, (B) L-rhamnose and (3) 3-*O*-methyl-D-glucose injected (filled symbols, solid lines) and gavaged (unfilled symbols, broken lines) into rats (right panels) and pigeons (left panels) were cleared from the blood rapidly and mono-exponentially. Insets show probe concentrations for the final three blood sampling times (t=90, 150, 240 min) on a semi-logarithmic plot. Values are means ± s.e.m. (N=6).

(Table 2). Fractional absorption was calculated for each carbohydrate by comparing AUCs from gavage trials to those from injection trials (Table 2). Pigeons absorbed significantly more L-arabinose and L-rhamnose than rats, and both rats and pigeons absorbed L-arabinose significantly more than L-rhamnose. The difference between L-arabinose and L-rhamnose was significant even after correction for their different molecular masses ($F_{1,10}$=29.3; P<0.001). Absorption of 3-*O*-methyl-D-glucose was nearly complete and did not differ significantly between pigeons and rats (Table 2).

Test for mediated uptake of probes

Because pigeons absorbed more L-arabinose and L-rhamnose than rats in the previous experiments, we used competitive inhibition tests to determine whether some absorption of these solutes might be mediated in pigeons but not in rats. In positive control experiments with both pigeons and rats, [^{14}C]D-glucose uptake by everted sleeves *in vitro* was significantly inhibited when sleeves were incubated with 100 mmol l^{-1} D-glucose compared with 100 mmol l^{-1} mannitol (the control) for both rats and pigeons (for rats: t_4=–55.1; P<0.001; pigeons: t_5=–24.3; P<0.001). In contrast, the uptake of putative paracellular probes was not significantly inhibited when tissues were incubated in the presence of either 100 mmol l^{-1} D-glucose or the solutes themselves (P>0.05), with two exceptions (Fig. 2). In laboratory rats, [^{3}H]L-rhamnose was significantly inhibited by 100 mmol l^{-1} D-glucose (t_5=–14.7; P<0.001), and in pigeons [^{3}H]lactulose was significantly inhibited by unlabeled lactulose (t_5=–3.0; P=0.016). There was no significant difference in total D-glucose uptake between rats and pigeons ($F_{1,8}$=0.62; P=0.45; Fig. 2).

Differences in absorption at tissue level and net water flux

Clearance of L-arabinose and L-rhamnose in perfused sections of intestine was about twice as high in pigeons as in laboratory rats ($F_{1,8}$=17.6; P=0.003), and L-arabinose was absorbed significantly more than L-rhamnose ($F_{1,8}$=27.9; P=0.001; Fig. 3). The difference between L-arabinose and L-rhamnose was significant even after correction for their different molecular masses ($F_{1,8}$=29.4; P=0.001). Despite the relatively low Na^+ concentration used in *in vivo* fractional absorption experiments (30 mmol l^{-1}), we found similar species differences in probe absorption in perfusion experiments when the Na^+ concentration was 70 mmol l^{-1}, which is adequate to support Na^+-coupled transport (Hopfer, 1987). There was no significant difference between pigeons and rats in absorption of D-glucose (respectively, 331±58 nmol min^{-1} cm^{-1} and 357±22 nmol min^{-1} cm^{-1}; t_5=–0.47; P=0.66).

Clearance of cellobiose and raffinose did not differ significantly between rats and pigeons ($F_{1,8}$=0.1; P=0.79) and was minimal compared to that of smaller probes (Fig. 3). Clearance of cellobiose was marginally significantly greater than for raffinose ($F_{1,8}$=5.0; P=0.06), and cellobiose clearance was significantly greater than zero in both species (pigeons: t_4=3.6; P=0.02; rats: t_4=6.6; P=0.003). Raffinose clearance was immeasurably low in both species; mean values did not differ significantly from zero (pigeons: t_5=0.2; P=0.88; rats: t_4=0.4; P=0.74; Fig. 3).

Under conditions where pigeons had significantly greater clearance of small probes, rats had significant net water absorption (mean net water flux=1.50±0.27 μl h^{-1} cm^{-1} (t_5=5.5; P=0.003) yet pigeons neither had net water absorption nor secretion (mean=–0.30±0.030 μl h^{-1} cm^{-1}; t_5=–1.0; P=0.37 for difference from zero). Rats had significantly greater net water absorption than pigeons (t_{10}=–4.4; P=0.001).

Test for difference in gut morphometrics

Pigeons had significantly shorter small intestines than rats (pigeons: 60.3±2.9; rats: 89.1±4.06; t_3=–14.7; P=0.001). The average circumference along the length of the small intestine was larger in the pigeon compared with the rat (pigeons: 1.16±0.06; rats: 1.05±0.01; t_3=3.83; P=0.03), but not enough to

Table 2. *Elimination rate constants (K_{el}) post-injection and post-gavage and fractional absorption (F) of probes in pigeons and rats*

Probe	Post-injection	Post-gavage	Effect of injection *vs* gavage and probe on K_{el}	Fractional absorption (F)	Effect of probe on F	Effect of Species on F
L-arabinose						
Pigeon	–0.020±0.006	–0.016±0.004		0.61±0.03		
Rat	–0.006±0.002	–0.009±0.001		0.34±0.04		
L-rhamnose					$F_{1,10}$=20.2; P<0.001	$F_{1,10}$=40.1; P<0.001
Pigeon	–0.014±0.002	–0.008±0.002	$F_{5,50}$=1.3; P=0.28	0.39±0.06		
Rat	–0.008±0.002	–0.011±0.002		0.23±0.02		
3OMG						
Pigeon	–0.013±0.004	–0.008±0.001		0.93±0.06		t_5=–0.4; P=0.72
Rat	–0.009±0.004	–0.005±0.001		0.93±0.04		

Values are means (min^{-1}) ± s.e.m.

3OMG, 3-O-methyl-D-glucose.

A repeated-measures ANOVA was used to determine if there was a significant difference in K_{el} post-injection *versus* post-gavage as well as among the three probes, and to determine if there was a significant effect of probe size (L-arabinose or L-rhamnose) or species on F. An independent t-test was used to compare F of 3OMG between species.

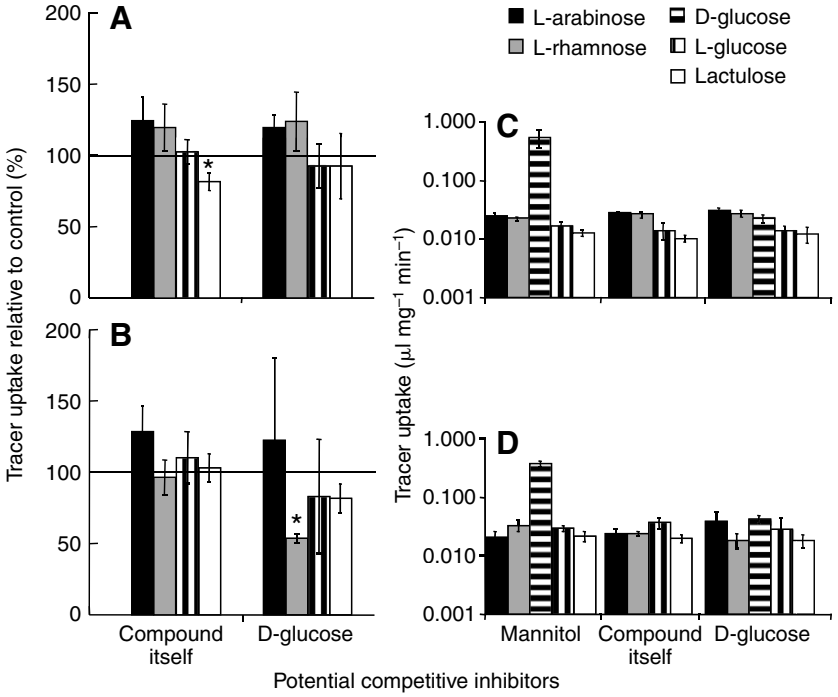

Fig. 2. (A,B) Uptakes (relative to control tissues) of tracer amounts of putative paracellular probes in everted sleeves of intestine were not significantly self-inhibited (100 mmol l^{-1} of the probes themselves) or inhibited by D-glucose (100 mmol l^{-1}). In pigeons (A), probe uptake normalized to control (100 mmol l^{-1} mannitol) was not significantly different from 100% in pigeons except that lactulose was significantly inhibited by itself. Significant inhibition of L-rhamnose (<100% of uptake for control treatment) was noted in rats (B) when 100 mmol l^{-1} D-glucose was present in solution. Asterisks indicate significantly less than 100% (P<0.02). Values are means ± s.e.m. (N=5–7 sleeves/treatment). (C,D) Raw uptakes (μl mg^{-1} min^{-1}) of tracer amounts of D-glucose and putative paracellular probes in the presence of unlabeled mannitol (control), the probe itself or D-glucose in pigeons (C) and rats (D). There was no significant difference in D-glucose uptake between rats and pigeons ($F_{1,8}$=0.62; P=0.45).

compensate for shorter length. Pigeons had significantly less small intestine nominal surface area than rats (pigeons= 68.6±5.3 cm^2; rats=93.1±3.7 cm^2; t_8=–3.4; P=0.01), and the mucosal surface enlargement factor was not significantly different between the species (pigeon=8.93±0.87; rat=7.45± 0.65; t_8=1.2; P=0.25). The number of small intestine enterocytes in pigeons and rats were not significantly different from each other (pigeons=$1.5\times10^8\pm2.1\times10^7$; rats=$1.6\times10^8\pm8.4\times10^6$; t_8=–0.4; P=0.69), but pigeons had a marginally greater density of enterocytes than rats (no. cells mm^{-1}; pigeons: 166±5.5; rats: 154±3.67; t_3=3.4; P=0.04).

Discussion

Our goal was to perform the first comparative study of paracellular absorption in a similar-sized bird and mammal using uniform methodology. We found that absorption of inert carbohydrates in intact pigeons was about double that in laboratory rats, consistent with previously reported differences among mammals and birds, and for the first time we were able to conclude that differences apparent at the whole animal level also occur at the tissue level. In the following sections we discuss potential mechanistic explanations. We finish with an overview of the functional significance of differences in paracellular absorption: in pigeons the majority of glucose absorption appears to be paracellular, whereas in rats this route contributes less than 10% of total glucose absorption.

Pigeons exceed rats in paracellular absorption at the whole-animal level

We hypothesized that pigeons would have greater absorption of inert carbohydrate probes compared to laboratory rats, based on previous studies of small birds (<200 g) that showed relatively efficient absorption (56–90%) of water soluble carbohydrates whose absorption is thought to be passive and not mediated, such as L-glucose (Afik et al., 1997; Caviedes-Vidal and Karasov, 1996; Karasov and Cork, 1994; Levey and Cipollini, 1996) and L-arabinose and L-rhamnose (Chediak et al., 2003). Indeed, intact pigeons absorbed twice as much L-arabinose and L-rhamnose as did intact laboratory rats. Because these molecules presumably permeate across the small intestinal mucosal epithelium primarily through the paracellular pathway (Powell, 1987), and because paracellular transport involves movement of compounds across the size-selective tight junction inbetween intestinal epithelial cells (Powell, 1987), we also expected, and found, that the smaller probe (L-arabinose, M_r=150) was absorbed more readily than the larger probe (L-rhamnose, M_r=164) in both species.

The greater absorption of L-arabinose and L-rhamnose that we

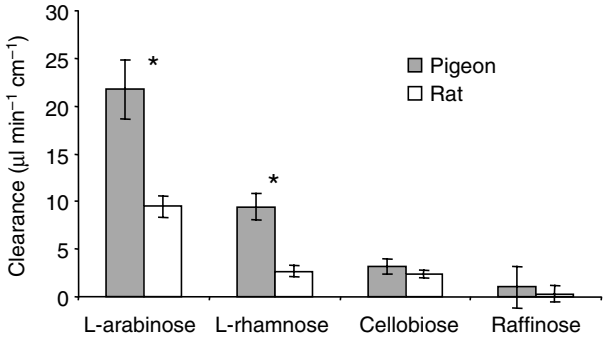

Fig. 3. Pigeons (filled bars) had significantly greater clearance (μl min^{-1} cm^{-1}) of L-arabinose and L-rhamnose than rats (open bars) in loops of intestine. Duodenal clearance (μl min^{-1} cm^{-1}) of larger probes (cellobiose: M_r=342.3; raffinose: M_r=594.5) was relatively low for both pigeons (filled bars) and rats (open bars). Raffinose clearance was negligible. Values are means ± s.e.m. (N=5–6 rats, 6 pigeons). See text for statistical comparisons.

measured in the pigeon as compared to the rat was not simply a general artifact of some difference in our experimental procedure with the two species We also measured the absorption of 3-*O*-methyl-D-glucose, a non-metabolizable, actively transported analogue of D-glucose that is absorbed both transcellularly and paracellularly. Absorption of this compound was high (93%) and not significantly different between pigeons and rats.

Higher absorption of inert carbohydrate probes by pigeons was not due to mediated absorption

The absorption of the carbohydrate probes was higher in pigeons compared to rats. If pigeons, but not rats, absorb the probes *via* active as well as passive processes, this could explain why pigeons have more extensive absorption of these carbohydrates than rats. In order to test this, we used everted sleeves to measure whether the uptake of the carbohydrate probes could be self-inhibited or inhibited by D-glucose. Lack of self-inhibition despite inhibition by D-glucose can occur if the transporters(s) (e.g. SGLT1, GLUT2) have higher affinity for D-glucose than for the respective probe. Either result would suggest that absorption of these probes is at least partially mediated. We found that in pigeons, uptake of L-arabinose and L-rhamnose was not competitively inhibited, but in rats, L-rhamnose uptake was significantly reduced when 100 mmol l^{-1} D-glucose was in the solution. Thus, we have no evidence of mediated absorption of the probes that were absorbed more extensively in intact pigeons or in perfusions of their intestines. If the rat does exhibit some mediated absorption of L-rhamnose, then the magnitude of the difference between rats and pigeons in the extent of paracellular absorption of L-rhamnose would be underestimated.

Additionally, we found no significant difference in total D-glucose uptake (active + passive) between rats and pigeons. Using a similar technique, mediated uptake rates of D-glucose were measured in pigeons (Obst and Diamond, 1989) and rats (Debnam et al., 1988). Using sleeves from the jejunum in a solution with 50 mmol l^{-1} D-glucose, mediated uptake rates in pigeon tissues were ~2 nmol mg^{-1} min^{-1} and in rat tissues ~375 nmol cm^{-1} min^{-1}. Assuming intestinal tissue is ~95 mg cm^{-1} (Debnam et al., 1988), then the rate of mediated absorption in the rat is about double that in the pigeon.

Pigeons exceed rats in paracellular absorption at the tissue level

Conceivably, the higher paracellular absorption in intact pigeons compared with rats is due to differences at the tissue level. It is also plausible that there are no tissue-level differences in absorption but that digesta are simply retained in contact with absorptive surfaces longer in the pigeon than in the rat. Permeability of paracellular probes at the tissue level has not been investigated in species with significant differences in paracellular absorption based on whole animal experiments. In our study, segments of perfused intestine indeed yielded comparable results to our experiments on intact animals. Like our experiments on intact animals, pigeons had double the clearance of L-arabinose and L-rhamnose than rats, suggesting that species variations in paracellular absorption that we noted at the whole animal level are not explained by gastrointestinal differences such as differential gut handling or residence time. This is the first study that we know of investigating differences in paracellular absorption *in vivo* at the tissue level between species. Pigeons do not have more extensive paracellular absorption than rats simply because there is more time for probes to be absorbed across tight junctions

We were also able to confirm that the difference in paracellular absorption between rats and pigeons in segments of intestine was not an experimental artifact of measuring high absorption in pigeons generally. Absorption of D-glucose was not significantly different between pigeons and rats. Our values for D-glucose absorption in rats were also comparable to other published values in rats perfused with a similar D-glucose concentration (Meddings and Westergaard, 1989).

Effective pore size in tight junctions does not seem greater in pigeons compared with rats

Enhanced probe flux across porous epithelia might be explained by increased effective pore size of the tight junction and/or increased paracellular solvent flux. In order to investigate whether differences in paracellular transport at the tissue level are due to species differences in the effective pore size in tight junctions, we also perfused the intestine of rats and pigeons with larger carbohydrate probes: cellobiose (M_r=342.3) and raffinose (M_r=554.5). Carbohydrate clearance declined with increasing M_r, but absorption became immeasurably low for both species at the same probe M_r, suggesting an equivalent molecular size cut-off for pigeons and rats and thus a comparable tight junction aperture.

Can differences in water flux explain the differences in paracellular absorption?

We found no difference in clearance of large-sized probes between pigeons and rats but a twofold difference in clearance of smaller probes (L-arabinose and L-rhamnose), which is consistent with higher solvent flow through the tight junctions in pigeons than in rats. However, when the small intestine of pigeons and rats were perfused with an isosmotic Ringer's solution containing 50 mmol l^{-1} D-glucose, rats, but not pigeons, had significant net water absorption. Our values of net water absorption in rats are comparable to fluxes measured in perfused rats in similar studies (e.g. Fagerholm et al., 1999; Sadowski and Meddings, 1993).

How could pigeons have comparable rates of D-glucose absorption as rats yet with no net water absorption if the majority of D-glucose absorption in pigeons is paracellular? Comparative measures of net water flux do not provide a strong test of the hypothesis that differences in solvent flux through the tight junction underlie differences in paracellular solute absorption. We used net water flux as a proxy for determining paracellular net fluid movement, but there is no known direct method to determine the pathway of water movement in the small intestine, and the molecular mechanisms and relative importance of paracellular and transcellular fluid movement are unclear. It is thought that water moves passively across the tight junction or the cell membrane due to osmotic gradients created by solute absorption (Masyuk et al., 2002), but there is only indirect evidence and theories to support transcellular (Loo et al., 1996; Meinild et al., 1998; Ramirez-Lorca et al., 1999;

Zeuthen et al., 2001; Zeuthen et al., 1997) or paracellular (Fromter and Diamond, 1972; Pappenheimer and Reiss, 1987; Powell, 1987) modes of fluid transport. In future studies, it would be interesting to compare differential expression of aquaporin isoforms (Ma and Verkman, 1999) between rats and pigeons as well as differences in water flux *via* SGLT1 (Zeuthen et al., 1997) between mammals and birds. Even if fluid transport occurs solely by the paracellular pathway, our measurements of net water flux may not indicate that rats have more extensive (paracellular) water absorption compared to pigeons. Measures of net water flux are only a proxy for net fluid movement across epithelia and can be confounded by other physiological processes such as differential ion absorption/secretion and unidirectional fluid movement.

Higher paracellular absorption in pigeons is not due simply to greater surface area or more tight junctions

Could small intestine morphological differences between the two species account for differences in paracellular absorption? Pigeons had significantly less small intestine nominal surface area than rats, which is consistent with broader comparisons that have been made between birds and non-flying mammals (Lavin, 2007). The mucosal surface enlargement factor was not significantly different between pigeons and rat, and our value of average rat mucosal surface enlargement factor is comparable to published values (e.g. Fisher and Parsons, 1950). The number of small intestine enterocytes (a proxy for the number of tight junctions) also was not significantly different between rats and pigeons. There was a trend for more absorptive surface per length of intestine in pigeons compared with rats (based on enterocyte density), but uptake of D-glucose was not significantly greater per unit length of intestine, suggesting that enhanced absorption is not a consequence. While pigeons had higher absorption of L-arabinose and L-rhamnose at both the whole animal level and in perfused intestinal segments, pigeons have less small intestine nominal surface area and comparable villus amplification and number of tight junctions than rats, suggesting that pigeons have higher tight junction permeability to carbohydrate probes.

Functional significance of differences in paracellular absorption

We used our data on intestinal paracellular permeability and total D-glucose absorption at 50 mmol l^{-1} to estimate the proportion of D-glucose clearance that was paracellular in pigeons and rats. We used the Renkin molecular sieving function (Renkin, 1954) and our clearance data on L-rhamnose to estimate paracellular permeability for D-glucose, which has a larger M_r and molecular radius than L-rhamnose. The Renkin function relates relative flux to the molecular radii of solutes in relation to an effective cylindrical pore radius of the tight junction. We used our values for L-rhamnose clearance and physiologically reasonable values for the effective pore size of the tight junction (minimum radius=7 Å; maximum radius=14 Å) based on human small intestine, which has an estimated pore radius of 8–13 Å (Fine et al., 1995). We also corrected for differences in L-rhamnose and D-glucose diffusivities in free solution using $M_r^{1/2}$ (Smulders and Wright, 1971) and used estimated molecular radii of 3.6 Å and 4.0 Å for L-rhamnose and D-glucose, respectively (Fagerholm et al., 1999; Hamilton et al., 1987). We estimated the minimum (pore size=7 Å) and maximum (pore size=14 Å) percentage of total D-glucose absorbed paracellularly to be 8±2% and 11±3% in rats and 42±12% and 59±16% in pigeons. Our method of estimating passive D-glucose absorption using data on L-rhamnose, which has a smaller molecular size is a more sophisticated way of correcting for differences in molecular size between probes than simply using the square root of the molecular mass. But even if we use that simpler approach, the conclusions hold. These might be slight overestimates if the absolute rate of mediated absorption is depressed due to anesthesia (Uhing and Kimura, 1995), but the estimate of the proportion of absorption that is paracellular in pigeons is high and comparable to other measurements in intact avian species: >80% in 120-g rainbow lorikeets [*Trichoglossus haematodus* (Karasov and Cork, 1994)], and >70% in 25-g house sparrows [*Passer domesticus* (Chang and Karasov, 2004)]. Based on our measurements in anesthetized, perfused pigeons and rats, combined with our findings in intact, whole animals, the extent of paracellular absorption of small water soluble nutrients such as monosaccharides and amino acids is likely high in the pigeon and at least double that of the rat. Differences in paracellular permeability are not limited to between species, but also within species [e.g. human diseased states (Vogelsang et al., 1998); human developmental stages (Beach et al., 1982)].

Although it is tempting to use a mathematical approach (e.g. the Renkin function or Pappenheimer's revised Kedem–Katchalsky model) to compare distributions of pores for specific probe sizes between species or to extrapolate probe clearances to get estimates for pore radii (and thus molecular radii at which probe clearance would be 0), we would have to make certain assumptions, for example that probe absorption is a consequence of diffusion or solvent drag (not both) through a porous membrane. Yet Fine et al. (1994) attributed passive absorption to both solvent drag and diffusion (but see above for caveats regarding the use of net water flux to study solvent drag). Furthermore, the Renkin model assumes a single pore with a single pore size, but some authors have suggested that there could be populations of more than one pore size or type (He et al., 1998). Differential pore sizes/types could be another mechanism explaining difference between rats and pigeons.

Another nutritional significance of our findings concerns absorption of water-soluble compounds such as toxins and naturally occurring secondary chemicals in foods (Harborne, 1993). An interesting future study could compare permeability of a toxin between these species. Our results suggest a rather similar effective molecular size discrimination in pigeons and rats, with relatively little absorption in either species of compounds larger than M_r of about 350 Da and molecular radius 4.8 Å. Note, however, that based on our measurements we cannot determine whether this arises because of features of effective pore radius and/or solvent drag. Thus, whereas there does not seem to be significant absorption for water-soluble solutes of M_r>350 at the whole animal level and at the tissue level, it is not clear whether comparable tight junction size discrimination between species accounts for this finding.

Our data are unique in that they suggest that differences in absorption of water-soluble compounds such as L-arabinose and

L-rhamnose that are observed in intact whole animals also occur at the level of the tissue. The lack of evidence for mediated absorption of either compound in the pigeon, and the pattern of size dependency in absorption that is consistent with molecular sieving, are also consistent with the idea that these compounds are absorbed by the paracellular route. The greater absorption in pigeons than in rats, is not explained by greater small intestine surface area or more tight junctions in the pigeon, and therefore implies greater paracellular permeability in the pigeon. But, until methods are developed to measure paracellular fluid flux directly, it remains uncertain whether the underlying mechanism(s) for the difference in paracellular absorption is enhanced fluid movement across the tight junction or greater tight junction effective pore size. Whatever the exact mechanism, the paracellular pathway of both species limits absorption to molecules in the size range of glucose and amino acids, and the pathway appears to account for the majority of D-glucose absorption in the pigeon, but less in the rat.

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