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SHORT COMMUNICATION

Intestinal paracellular absorption is necessary to support the sugar oxidation cascade in nectarivorous bats

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ABSTRACT

We made the first measurements of the capacity for paracellular nutrient absorption in intact nectarivorous bats. *Leptonycteris yerbabuenae* (20 g mass) were injected with or fed inert carbohydrate probes L-rhamnose and D(+)-cellobiose, which are absorbed exclusively by the paracellular route, and 3-*O*-methyl-D-glucose (3OMD-glucose), which is absorbed both paracellularly and transcellularly. Using a standard pharmacokinetic technique, we collected blood samples for 2 h after probe administration. As predicted, fractional absorption (f) of paracellular probes declined with increasing M_r in the order of rhamnose (f=0.71)>cellobiose (f=0.23). Absorption of 3OMD-glucose was complete (f=0.85; not different from unity). Integrating our data with those for glucose absorption and oxidation in another nectarivorous bat, we conclude that passive paracellular absorption of glucose is extensive in nectarivorous bat species, as in other bats and small birds, and necessary to support high glucose fluxes hypothesized for the sugar oxidation cascade.

KEY WORDS: Bioavailability, D-Cellobiose, Intestine, *Leptonycteris yerbabuenae*, L-Rhamnose, Nutrient absorption, Sugars, 3-*O*-methyl-D-glucose

INTRODUCTION

The capacity to fuel exercise metabolism entirely using sugar from nectar while hovering is a notable feature of nectarivorous birds and bats that is made possible through the operation of a pathway aptly named the 'sugar oxidation cascade' (Suarez et al., 2011). The cascade refers to the path of carbon from flowers through the digestive system all the way into the mitochondria. In the gut, the high glucose flux is supported by high intestinal sucrase activities and a high capacity for glucose absorption (Suarez et al., 2011). In hummingbirds, transcellular glucose absorption mediated by membrane transport proteins is insufficient to account for the flux, and hummingbirds rely on passive non-mediated intestinal nutrient absorption to meet the majority of their metabolic demands (Suarez et al., 2011). Our calculations indicate that the same must be the case for nectarivorous bats, though certain necessary whole-animal measurements have never been made in any nectarivorous bat species. For example, Suarez et al. (2011) found that hovering Pallas' long-tongued bats (*Glossophaga soricina* Pallas 1766) had an oxygen consumption rate of 24.48 ml O_2 g^{-1} h^{-1}, 80% of which was from the oxidation of dietary sugar, implying an intestinal monosaccharide absorption rate of 2.22 µmol g^{-1} min^{-1}. We do not know the bat's maximal mediated uptake rate of glucose, but even assuming (i) the highest value measured in a vertebrate, that for a hummingbird (0.6 µmol min^{-1} cm^{-2}; McWhorter et al., 2006), and (ii) the bat's likely intestinal nominal surface area (0.5 cm^2 g^{-1} body mass for a bat of its size; Price et al., 2015a), mediated uptake could account for only about 14% of the intestinal flux. In other bats, intestinal paracellular sugar absorption rates are high and account for the majority of D-glucose absorption (Price et al., 2015a), but whole-animal measures of paracellular absorption have never been made in intact nectarivorous bats.

The goal of the present study was to test, for the first time, the capacity for paracellular nutrient absorption in an intact nectar-eating specialist bat species, the Saussure's long-nosed bat (*Leptonycteris yerbabuenae*; Martínez and Villa-R, 1940). To assess paracellular absorption capacity, we used two inert carbohydrates that bracket the relative molecular mass (M_r) of sugars absorbed in nature (e.g. glucose and fructose, M_r=180). Our probes, L-rhamnose (M_r=164) and D-(+)-cellobiose (M_r=342), are both absorbed passively exclusively by the paracellular route. For comparison, we also measured absorption of the non-metabolizable D-glucose analog 3-*O*-methyl-D-glucose (3OMD-glucose, M_r=194), which is absorbed both paracellularly and transcellularly by a transporter(s). As paracellular absorption declines with increasing molecular size of the solute because of the pathway's sieve-like qualities (Chediack et al., 2003), we predicted a molecule size dependence for absorption of the order of L-rhamnose (M_r=164)>D(+)-cellobiose (M_r=342). Additionally, if the majority of glucose is absorbed passively by the paracellular route in nectar-eating bats, as in bats with other diets (Price et al., 2015a), then paracellular absorption will be extensive and high enough to account for the majority of absorption of 3OMD-glucose. We predicted that the capacity for paracellular nutrient absorption in this nectarivorous bat species would be as high as that observed in other bats and in birds.

MATERIALS AND METHODS

Study site and sample collection

Twenty adult male Saussure's long-nosed bats (*L. yerbabuenae*) were collected in the Chamela region in the central Pacific coast of Mexico (approximately (19°22′–19°35′ N, 104°56′–105°03′ W). This bat consumes nectar and pollinates the flowers of 22 plant species at this site (Stoner et al., 2003). Bats were captured using mist nets and handled under permit (Oficina de Fauna Silvestre, Mexico, SGPA/DGVS/00268/13) and according to humane handling guidelines approved by the American Society of Mammalogists. After capture, bats were weighed (±0.1 g), individually bagged, and

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transported to the field laboratory. Measurements on intact bats were begun within 2 h of capture.

Sugar absorption experiments

We prepared one probe solution containing 30 mmol l^{-1} L-rhamnose, 100 mmol l^{-1} D(+)-cellobiose, 75 mmol l^{-1} 3OMD-glucose, and 35 mmol l^{-1} NaCl and 30 mmol l^{-1} L-arabinose to make the solution 305 mOsmol l^{-1} (isosmotic with bat blood). The oral and injected doses differed in volume; we fed 2% of body mass (ca. 400 µl for 20 g bat) and injected 0.5% of body mass intraperitoneally (i.p., ca. 100 µl for 20 g bat). The amount of liquid given for both treatments was measured by weighing the syringe (±0.001 g) before and after administration of the probe solution. We assigned one of the two treatments (either oral administration or i.p. injection) to a single bat. Seven blood samples of ca. 28 µl/sample were taken serially 7, 15, 30, 45, 60, 90 and 120 min after administration of the probe solution. Total blood removal was ca. 200 µl, which is 10% of blood volume, assuming 10% of body mass is blood. Plasma was separated immediately using a standard hematocrit centrifuge and stored frozen at −80°C until analysis in the laboratory. Each bat was released after its trial was completed (about 2 h).

Quantification of plasma probe concentration

Plasma samples were analyzed by high-performance liquid chromatography (HPLC) in an Agilent 1100 series Hewlett Packard machine. Blood samples were first derivatized by reductive amination with anthranilic acid (2-aminobenzoic acid), following Du and Anumula (1998) with minor modifications. Briefly, plasma samples were mixed with 50 µl of anthranilic acid reagent solution (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 heated at 70°C for 3 h. After they had cooled to ambient temperature, we added 300 µl of HPLC solvent A (see below) to vials, which were mixed vigorously to expel the hydrogen gas evolved during the derivatization reaction.

Carbohydrate derivatives were separated on a Waters Pico Tag® C-18 reversed phase HPLC column (3.9×150 mm, 5 µm; part number WAT088131; Waters Corporation, Milford, MA, USA) using a 1-butylamine–phosphoric acid–tetrahydrofuran mobile phase system. The separations were performed at 23°C using 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 and solvent B consisted of equal parts solvent A and HPLC-grade acetonitrile.

Derivatives of carbohydrate probes were detected with a Perkin-Elmer LS 50 B luminescence spectrometer (excitation wavelength 360 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). Chromatograms were compared with standards for identification of individual sugars.

Pharmacokinetic calculations

Pharmacokinetic calculations were done following Fasulo et al. (2012). In brief, for each molecule, the concentration in each plasma sample at time t was normalized to the mass of each sample (C_t, nmol g^{-1} plasma) and to the administered dose (nmol), and plotted against sampling time since the molecule was administered either orally or by injection. The resultant values have units of g^{-1} plasma. The integration of the area under this curve (AUC_t) represents the amount of molecule that has been absorbed from time 0 up to time t, whereas AUC_{total} denotes the total amount of molecule absorbed from 0 until infinity (∞). Following typical procedures in pharmacokinetics, the area from t=0 to t=X min (when the final blood sample was taken) was calculated using the trapezoidal rule. The area from t=X min to t=∞ was calculated as $AUC_{X\rightarrow\infty}=C_t$ (at t=X)/k, where k is a rate constant that can be determined for each bat in each experiment based on the terminal portion of its absorption curve. The total $AUC_{0\rightarrow\infty}$ was obtained by summing the two areas. Fractional absorption (f), or bioavailability, for each compound was estimated based on the ratio between the area under the curve of plasma concentration versus time for oral administration experiments (AUC_{oral}, in units of min g^{-1} plasma) and injection experiments ($AUC_{injection}$): $f=AUC_{oral}/AUC_{injection}$. This method of calculating f is favored because it makes no major assumptions about compartments or kinetics. Fractional absorption estimates how much of the ingested probe was absorbed into the animal's system. The calculations of AUCs and their statistical comparison (below) were performed based on data for 75% of individuals, although data shown in figures are mean values for all individuals. In the former instances, semilog plots of plasma probe concentration versus time were inspected for each probe in each individual and were excluded from calculations when semilog plots were outside the range $-1<r<-0.6$. Apparent f for each individual bat could not be calculated because individuals were not administered probes by both routes of administration. However, mean and variance of f for the population of bats was estimated based on the mean and variance for each route of administration as described in Stuart and Ord (1994). This method assumes that the oral and injection data were independent, which was the case because different sets of bats were used.

Statistical analysis

Numerical data are presented as means±s.e.m. (N=number of animals). Comparisons of f with unity were performed using a one-tailed t-test [t_{N-1}=(1−mean f)/s.e.m.]. AUCs, terminal slopes and intercepts for the three molecules, measured within each bat, were analyzed by repeated measures ANOVA. Terminal slopes for plasma probe concentrations post-injection were compared with those post-oral administration using analysis of covariance (ANCOVA) with time since probe administration as the covariate. The F-values of ANOVA/ANCOVA are presented in the text with the relevant degrees of freedom as subscripts. Statistical significance was accepted for P<0.05.

RESULTS AND DISCUSSION

Plasma concentration of 3OMD-glucose declined post-injection, as it did post-oral administration after a lag of 15 min (Fig. 1A). The terminal slopes on a semilog plot for the time points at 15 min post-administration and later did not differ significantly by route of administration for 3OMD-glucose (ANOVA, $F_{1,105}$=0.02, P>0.8; Fig. 1B, Table 1) or for L-rhamnose ($F_{1,105}$=0.23, P>0.6; Fig. 1D, Table 1). The data for D(+)-cellobiose were not as consistent (Fig. 1E,F), and the semilog plots marginally declined with time post-administration ($F_{1,105}$=3.26, P=0.073) and marginally differed with route of administration ($F_{1,105}$=2.86, P=0.094). The curve of plasma probe concentration versus time since oral administration was closest to the curve for injection in the case of 3OMD-glucose (Fig. 1A) and was farthest for cellobiose (Fig. 1C), indicating that relative absorption of the three probes ranked in the order 3OMD-glucose>L-rhamnose>D(+)-cellobiose, as predicted.

We used a subset of the data (75%; see Materials and methods for inclusion criteria) for a quantitative assessment of absorption.

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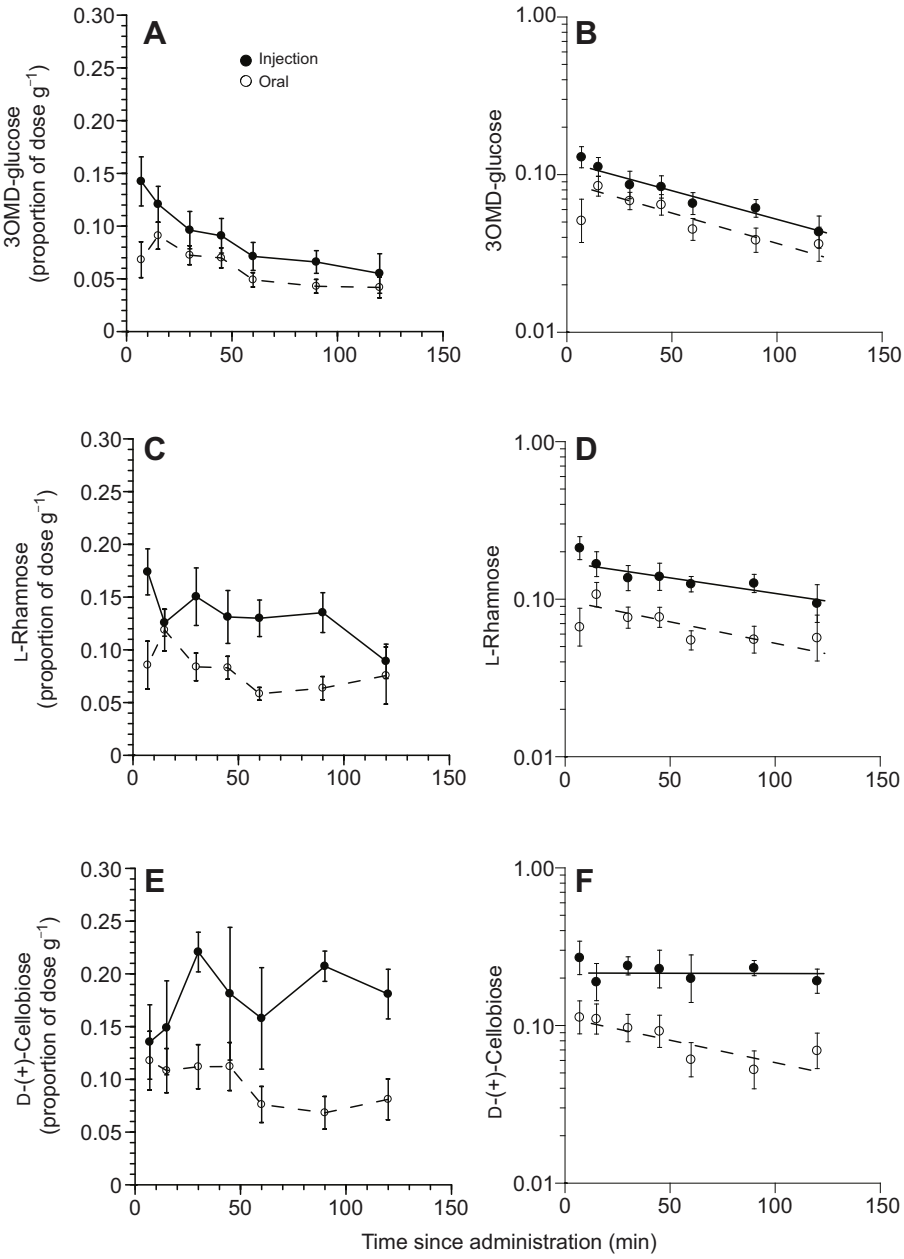

Fig. 1. Levels of carbohydrate probes in the plasma of the nectarivorous bat *Leptonycteris yerbabuenae* following oral or i.p. administration. Left-hand figures are plasma concentrations of (A) 3-*O*-methyl-D-glucose (3-OMD-glucose; proportion of dose per gram plasma), (C) L-rhamnose and (E) D-(+)-cellobiose in 10 bats administered the probes orally (open circles, dashed line) and 10 bats injected with the probe (filled circles, solid lines). Data are means±s.e.m. Right-hand figures are corresponding semi-log plots of plasma concentrations for the same data: (B) 3OMD-glucose, (D) L-rhamnose and (F) D-(+)-cellobiose. Lines shown in the right-hand figures are linear least-squares regression fits beginning 15 min post-administration, and data are means±s.e.m.

Average correlation coefficients for the log-linear decline of probe molecules within individual bats for injection trials averaged −0.93 for 3OMD-glucose-injected bats (N=9), −0.88 for L-rhamnose (N=7), and −0.73 for D(+)-cellobiose (N=5). Among bats administered the probes orally, the correlation coefficients were, respectively, −0.91 (N=9), −0.81 (N=9) and −0.86 (N=6). Slopes

Table 1. Data derived from plots of plasma probe concentration in individual bats administered probes either orally or by injection

	Slope of log-linear decline post-administration			AUC		
	3OMD-glucose	L-Rhamnose	D-(+)-Cellobiose	3OMD-glucose	L-Rhamnose	D-(+)-Cellobiose
Oral						
N	9	9	6	9	9	6
Mean	−0.00499	−0.00404	−0.006307	15.29	26.45	21.63
s.e.m.	0.00053	0.00047	0.001558	3.29	4.81	5.89
Injection						
N	9	7	5	9	7	5
Mean	−0.004482	−0.004513	−0.002744	19.48	46.52	124.63
s.e.m.	0.00065	0.000770	0.000686	1.94	9.31	34.55
P-value	*P*>0.5	*P*>0.5	*P*=0.061	*P*>0.2	*P*=0.04	*P*<0.006

AUC, area under curve.
P-values are for comparison of oral versus injection, by *t*-test.

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for log-linear decline did not differ between bats that were injected or were orally administered the probes (Table 1). AUCs calculated for oral administration trials (AUC_{oral}) were significantly lower than AUCs from injection trials ($AUC_{injection}$) for L-rhamnose and D-(+)-cellobiose, but not for 3OMD-glucose (Table 1). Calculated fractional absorptions (f=AUC_{oral}/$AUC_{injection}$) were 0.85±0.19 for 3OMD-glucose (not significantly different from unity; t_8=0.79, P=0.2), 0.71±0.14 for L-rhamnose (significantly lower than unity; t_8=2.07, P=0.04) and 0.23±0.06 for cellobiose (significantly lower than unity; t_5=12.8, P<0.001).

We found that in the nectarivorous bat *L. yerbabuenae*, rhamnose absorption was three times greater than that of cellobiose (71% versus 23%). Carbohydrates and amino acids can be absorbed by the passive paracellular pathway through the tight junctions between adjacent enterocytes (Price et al., 2015a). Because the size-sieving effect of tight junctions can affect the absorption of different-sized molecules (Chediack et al., 2003), these results were expected based on the larger molecular size of D-(+)-cellobiose (M_r=342 Da), the movement of which is more restricted than that of the smaller L-rhamnose (M_r=164 Da) as it passes through the tight junctions. A similar pattern of size-based differences in nutrient-sized molecule absorption has been found in birds and other bat species (Price et al., 2015a).

Our whole-animal measurements on *L. yerbabuenae* are consistent with those made by intestinal perfusion *in situ* in nectarivorous *G. soricina* (Price et al., 2015b), and the data on both species and methodologies underscore the importance of paracellular absorption in supporting the sugar oxidation cascade in nectarivorous bats. In the perfusion study, paracellular sugar absorption rates were high and could account for an estimated 93% (±10%, N=2; E.R.P., personal observation) of total 3OMD-glucose absorption at a luminal concentration of 10 mmol l^{-1}, and this percentage would be higher at higher, more transporter-saturating luminal concentrations. In the current study, the measured fractional absorption of L-rhamnose (71%) relative to that of 3OMD-glucose (85%) is consistent with paracellular absorption accounting for approximately 84% of D-glucose absorption (=100×71/85), similar to that in the perfusion study (see also Price et al., 2015a, for analogous findings in other frugivorous bat species). Moreover, the measured permeability of the *in situ* perfused intestine was high enough to account for the rate of glucose oxidation during hovering flight, which was about 2.2 µmol min^{-1} g^{-1} body mass (see Introduction). The passive clearance of the paracellular probe during *in situ* perfusion corresponded to a D-glucose clearance of 12.9 µl min^{-1} cm^{-2} (Price et al., 2015b), or 6.45 µl min^{-1} g^{-1} body mass, assuming 0.5 cm^2 g^{-1} body mass for a bat of its size (Price et al., 2015a). Thus, the necessary luminal glucose concentration for passive absorption rate to equal the rate at which dietary glucose was apparently absorbed and oxidized would be 0.34 µmol $µl^{-1}$ (=2.2 µmol min^{-1} g^{-1}/6.45 µl min^{-1} g^{-1}), or 340 mmol l^{-1} glucose. Calculations would be similar for fructose (with a similar M_r to glucose), which is relevant because the dietary sugars used in these studies include D-glucose, D-fructose, and sucrose, which yields equal amounts of glucose and fructose upon hydrolysis, the latter of which may be catabolized directly or converted to glucose (Suarez et al., 2011). Although we do not know the intestinal luminal sugar concentration in the bats, a luminal concentration of 340 mmol l^{-1} sugar or higher seems entirely reasonable considering the 15–20% sugar concentrations the bats were typically fed (yielding ~800–1100 mmol l^{-1} monosaccharides). Thus, as is the case in nectarivorous birds, digestion studies in nectarivorous bats are consistent with the idea that the majority of glucose absorption occurs by the paracellular pathway, and the measured epithelial fluxes and the calculated epithelial permeabilities in nectarivorous bats are consistent with the high glucose fluxes hypothesized for the sugar oxidation cascade.

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Competing interests
The authors declare no competing or financial interests.

Author contributions
N.R.-P., E.R.P., E.C.-V. and W.H.K. were involved in the conception and design of the study. N.R.-P. was involved in collecting the experimental data. N.R.-P. and C.M.F.-O. were involved in analyzing the samples. N.R.-P., E.R.P., C.M.F.-O. and W.H.K. were involved in interpreting the experimental data. N.R.-P. and W.H.K. wrote the manuscript with editorial input from E.C.-V., E.R.P. and C.M.F.-O.

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