Intestinal Nutrient Uptake Measurements and Tissue Damage:
Validating the Everted Sleeves Method

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ABSTRACT
The reliability of methods for nutrient uptake measurements across the intestinal epithelium relies on the integrity of the mucosal epithelium and the enterocytes. We tested effects of tissue handling during the "everted sleeves method" on the length of intestinal villi, the surface magnification, the circumference of the gut, and the thickness of the muscle layer in sunbirds (Nectarinia ossa), chicken (Gallus gallus), and mice (Mus domesticus). The sunbird has thin and delicate intestinal villi that are greatly affected by the everted sleeves method. After eversion and incubation, villi lost 30% of their original length. The severe tissue damage coincides with uptake measurements for glucose that were an order of magnitude lower than in other nectar-feeding (nectarivorous) birds of similar body size. Tissue handling during the everted sleeves method had significant effects on morphometric parameters of chicken and mouse intestines, but on a light-microscopical level, the tissue integrity and the cytology of the enterocytes were not altered. Therefore, we think that the everted sleeves method renders reliable and reproducible measurements of nutrient uptake in those species. We conclude that a histological evaluation is necessary to assess the reliability of the method before it is applied to adults or to the developmental stage of any species.

Introduction
The correct measurement of intestinal brush-border uptake rates for various nutrients in mammals, birds, and "reptiles" (nonavian sauropods) is pivotal in numerous research projects concerned with digestive function, nutritional ecology, and functional plasticity of the intestine. The introduction of a simple method to measure intestinal brush-border nutrient uptake in vitro (everted sleeves method) by Karasov and Diamond (1983) stimulated many physiological and ecological studies comparing intestinal nutrient uptake rates under various ecological and physiological conditions. The enterocytes of the mucosal epithelium provide the structural basis of nutrient uptake. The membrane of their microvilli carries the nutrient transporters (Ganapathy et al. 1994; Gardner et al. 1994), and the tight junctions between the enterocytes provide a barrier against uncontrolled flux from the gut lumen into the gut tissue (Ma et al. 1993; Ballard et al. 1995). Thus, the measurement of carrier-mediated brush-border nutrient uptake rates as well as diffusion mediated uptake by the everted sleeves method relies on a fully functional mucosal epithelium and cytologically intact enterocytes. Damage to the enterocytes or the mucosal epithelium reduces the density of membrane-bound transporters for nutrient uptake and opens the tissue for uncontrolled flow. Consequently, tissue damage will flaw the results of the measurement.

Results from the everted sleeves uptake measurements in a particular species have been reproduced reliably in many studies. However, for some species the measured uptake rates were surprisingly low (Cavides-Vidal and Karasov 1996; Karasov et al. 1996; Afik et al. 1997), and the variances of the measured uptake rates were exceedingly high and difficult to explain (Konarzewski and Starck 2000). Potentially, a yet-unidentified factor in the protocol may have affected the results. Although the technique relies on the integrity of the intestinal villi and the mucosal epithelium, a possible effect of the everted sleeves method on their histology has not been tested. Tissue handling during the everted sleeves procedure involves several steps that are critical for the cytological integrity of the mucosal epithelium: (1) eversion of excited sleeves of the gut, (2) 5-min preincubation in a medium (Ringer's solution), and (3) 1-2 min incubation in a stirred medium containing radioactively labeled nutrients. In this study, we evaluate effects of tissue handling on epithelial integrity and uptake rates by sampling intestinal tissue from birds and mammals before eversion, after eversion but before incubation, and after eversion and incubation. Dam-
age during tissue handling should appear in histological evaluations of tissue and in inflated variances or reduced uptake measurements of some individuals.

Material and Methods

Animals

Fifteen adult sunbirds, *Nectarinia osa* (5.6 ± 0.2 g SEM) were captured in June and July 1997 on the campus of Haifa University at Oranienbaum, Israel. The birds were housed in separate cages (40 × 30 × 25 cm) indoors at a constant temperature (24° ± 1°C) and day length (12 h) with sugar solution ad lib. Six birds were used to measure nutrient uptake rate, five for histology measurements, and the others in experiments to refine procedures. Swiss Webster laboratory mice were obtained from local breeding stock. The mice were housed in individual cages with commercial rodent pellets and water ad lib. Three mice were used to measure nutrient uptake rate. Gut tissue samples from the same animals were used for the histological measurements. Chickens were obtained from a local breeding farm at the age of 4 wk and housed in individual cages with commercial feed and water ad lib. We used three chickens for histological measurements.

Uptake Measurements

We measured mediated (i.e., active) uptake of D-[6,2H(N)] glucose (NEN, Boston) and total (i.e., mediated plus passive) uptake of L-[3H(U)] proline (NEN, Boston) into the tissue across the brush-border membrane as described in Karasov and Diamond (1983). In brief, 1-cm sleeves of everted tissue were preincubated in 40°C Ringer’s solution and suspended for 1 or 2 min above a stir bar spinning rapidly in a solution containing labeled 50-mmol D-glucose or 50-mmol L-proline. These incubation times were chosen because in other avian species they are sufficient to allow adherent fluid to equilibrate with labeled markers in the bathing solution, and uptake rates were detectable and still linear with time (to ensure measurement of unidirectional flux; Karasov and Weary 1990; Weary and Karasov 1992). Tissues were then rinsed or blotted, removed from the rod, weighed, incubated in a tissue solubilizer (Solunece-350, Packard, Meriden, Conn.), and counted in scintillation cocktail (Insta-Gel II Plus, Packard) on a Packard Tri-Carb 2100 TR scintillation counter (Packard). To correct for passive uptake and nonabsorbed nutrients in adherent mucosal fluid, we used tracer concentrations of nonactively absorbed L-[1,2-3H] glucose (NEN, Boston) or membrane-impermeable marker (1,2,3-H polyethylene glycol [PEG], MW = 4,000, NEN, Boston). Counts for each isotope were corrected for variable quench and for counts of the alternate isotope appearing in the same counting channel.

In sunbirds, the intestines were short (6.4 ± 0.2 cm), so we prepared only four sleeves from each of six birds. The two most proximal sleeves and the most distal sleeve were used to measure D-glucose uptake, and the other sleeve measured L-proline uptake. Uptakes of 50 mmol D-glucose were measured in three intestinal regions: proximal (first 10% of length), middle (45%–55%), and distal (last 10%) small intestine; uptake of L-proline was measured in the middle region only. Uptakes of D-glucose and L-proline were measured at 50 mmol because this concentration nearly saturates the transporters, and measured rates are thus near maximal (Levy and Karasov 1992). Summed D-glucose uptake capacities over the entire small intestine were calculated by interpolating uptakes per centimeter linearly between adjacent sections and integrating over the entire small-intestinal length.

Histology

To evaluate the effects of tissue handling, we sampled tissue of the intestine (1) before eversion, (2) after eversion but before incubation, and (3) after eversion, preincubation for 5 min, and incubation for 1 min in a stirred medium (preincubation medium is the same used for uptake measurements but without radioactive tracers). Tissue samples of three adult sunbirds, mice, and chicken were preserved in 5% paraformaldehyde in 0.1 mol phosphate-buffered saline at pH 7.4 and 4°C for at least 48 h. Before embedding, tissue samples were washed in a phosphate buffer and dehydrated through a graded series of ethanol to 96% ethanol. Following dehydration, tissue samples were embedded in hydroxyethyl methacrylate (Historesin, Leica Instruments, Wetzlar, Germany). From each tissue sample we sectioned 50 sections of 2-μm thickness. Sections were mounted on slides and stained with methylene-blue thionine. Microphotographs were taken using a Zeiss Axiosplan photomicroscope and Agfa APX100 film.

Morphometry

Sections were studied using a Jenaval Research Microscope connected to a video camera and a PC-based image analysis and morphometry system (SigmaScanPro, version 4.0, Jandel Scientific, SPSS, Chicago). From each section, we measured the circumference of the gut, the thickness of the muscle layer (tunica muscularis), and the height of the villi from the muscle layer to the top of the villus. For chicken and mouse, we measured the absorbptive surface magnification at different segments of the gut directly on digitized images by tracing the length of the brush border over a given arc of the circumference of the intestine. From each species, we took tissue samples from three individuals and measured 10 sections per tissue sample. We took 15 measurements of thickness of muscle layer and height of villi per section, resulting in 150 measurements per individual and 450 measurements per species. For surface magnification, we took one measurement per section, i.e., 15 measurements per individual and 45 measurements per species.
actions between treatment and individual animal were included as effect into the model. The $P < 0.05$ level was considered significant.

**Results**

**Uptake Measurements**

In sunbirds, intestinal masses of the 1-cm sleeves were, from proximal to distal, 24.6 ± 1.9 mg cm$^{-1}$, 20.3 ± 2.5, 13.7 ± 1.2, and 13.1 ± 1.1 ($N = 5$–8 sleeves from eight birds). In five birds for which we had measures in all positions, the effect of position on mass was significant ($F_{4,13} = 5.72$, $P = 0.01$). Mediated D-glucose uptake rates at 50 mmol were 34 ± 7 nmol min$^{-1}$ cm$^{-1}$ ($N = 5$ birds) and 30 ± 6 nmol min$^{-1}$ cm$^{-1}$ ($N = 6$) in the two most proximal sleeves and 19 ± 4 nmol min$^{-1}$ cm$^{-1}$ ($N = 4$ birds) in the most distal sleeve. In four birds for which we had measures in all positions, the effect of position on D-glucose uptake rate was not significant ($F_{4,6} =$ 1.48, $P = 0.3$). However, we had low power to detect such a difference. L-proline uptake at 50 mmol in the third intestinal position was 108 ± 18 nmol min$^{-1}$ cm$^{-1}$ ($N = 5$ birds).

The measures of maximal mediated D-glucose uptake (see above) were an order of magnitude lower than those reported for similar-sized nectarivorous rufous hummingbirds (Selasphorus rufus; 200–400 nmol min$^{-1}$ cm$^{-1}$ in the proximal two-thirds of intestine) with the use of the same technique. The L-proline uptakes were higher than in hummingbirds (40–65 nmol min$^{-1}$ cm$^{-1}$). To confirm that the relatively low D-glucose uptake rates in sunbirds were not due to some unidentified factor in our protocol, on one day we also measured uptake in Swiss Webster laboratory mice using exactly the same solutions as for sunbirds on those days. Uptakes in mouse middle small intestine, 694 ± 132 nmol min$^{-1}$ cm$^{-1}$ ($N = 3$ mice; two measures in each), were an order of magnitude higher than in sunbirds and similar to those reported earlier. Thus, the relatively low uptake rates observed in sunbirds do not appear to result from methodological differences with other studies using the everted sleeve technique. They are either an inherent feature of sunbird intestinal tissue or a result of how the sunbird intestinal tissue interacts with the everted sleeve method for measuring solute uptake.

**Histology and Morphometry**

The histology of the noneverted gut of sunbirds shows long and delicate villi (Fig. 1A; Table 1). The connective tissue core of the villi (lamina propria mucosae) is characterized by large blood vessels and extensive lymphatic spaces. Only a few, if any, smooth muscle cells can be found in the villi. Using light microscopy, we were not able to determine the lamina muscularis mucosae, which is either absent or so small that transmission electron microscopy would be required to detect it. The mucosal epithelium (lamina epithelialis mucosae) shows...
the typical characteristics of a single-layered epithelium with high prismatic cells. A high number of goblet cells occurs interspersed among the enterocytes, which are characterized by large vesicles. The layer of intestinal crypts consists of one to three crypts. The muscular wall (tunica muscularis) of the intestine is very thin (Table 1). The circular muscle layer is prominent, but the longitudinal muscle layer is barely recognizable.

Eversion of the gut had considerable effects on histology. About half of the length of the villi was crumpled up, mostly wrinkled and sticking together (Fig. 1B). The histological integrity of the villi was heavily affected. At some positions, the distal tip of the villi was ruptured and blood vessels and lymphatic spaces were torn open. The basal part of the villi, the layer of intestinal crypts, and the muscular wall were not affected (Fig. 1B). Incubation of the tissue samples in a stirred medium following eversion further increased tissue damage (Fig. 1C). In all sections, the distal part of the villi was simply shaved off. Only the very basal part of the villi, the layer of intestinal crypts, and the muscular wall of the gut have resisted the treatment. The few remaining enterocytes appear pale and without vesicles so that it seems that their cytoplasm has undergone changes, too.

We measured height of the villi, thickness of muscular wall of the gut, and circumference of the gut before treatment, after eversion, and after eversion and incubation. We did not measure absorptive surface in sunbirds because of the shaving effect on villi through incubation. For villi length, treatment was the only significant effect (length: \( F_{2,14} = 64.07, P < 0.0001 \)). The circumference of the gut and the thickness of the muscle layer were affected by treatment (circumference: \( F_{2,2} = 64.9, P < 0.0001 \); thickness: \( F_{2,3} = 131.8, P < 0.0001 \)) and individual circumference: \( F_{2,2} = 9.6, P < 0.0001 \); thickness: \( F_{2,2} = 247.8, P < 0.0001 \)). Section and measurement had no significant effect on villus length (Table 1). The same procedure was repeated with tissue samples from intestines of adult mice and chicken. The villi of the chicken gut are rather stout and solid (Fig. 2A) relative to those of the sunbird. The connective tissue core of the villi contains a considerable number of smooth muscle cells. Blood vessels are small and lymphatic spaces cannot be detected using light microscopy. The lamina muscularis mucosae can clearly be distinguished, and the tunica muscularis shows a distinct separation into longitudinal and circular fiber compartments. After eversion, we could not detect any significant tissue damage. The intestinal villi appeared to be broader and the epithelium appeared notched/dentilated, probably through stretching of the tissue. The epithelium and the enterocytes, however, were still intact (Fig. 2B). Eversion had no effect on villus height (Table 1), but the thickness of the muscle layer decreased while circumference of the gut increased. Consequently, the surface magnification of the mucosal epithelium was increased by eversion.

After eversion and incubation in a stirred medium, the villi and its epithelium still appeared intact, though compressed (Fig. 2C). Villus length decreased significantly (Table 1; ANCOVA, \( F_{1,13} = 121.9, P < 0.0001 \)) as compared with the untreated intestines or those that were only everted. A significant treatment effect on the thickness of the muscle layer was also found (Table 1; ANCOVA, \( F_{1,13} = 2.99, P = 0.05 \)). Treatment effects on circumference of the gut were highly significant (Table 1; ANCOVA, \( F_{1,13} = 104.3, P < 0.0001 \)), and the circumference increased further. When everted and incubated, the surface magnification decreased compared with eversion only. However, it was still larger than in the untreated state. The observed

| Table 1: Least squares means from comparisons of effects of everted sleeves method |
|-----------------------------------|-----------------|-----------------|-----------------|
| Species (\( N = 3 \))             | Untreated       | Everted         | Everted and Incubated |
| Sunbird:                          |                 |                 |                 |
| Villus height (mm) ...........    | 150             | 4.416 (±1.12)*  | 3.956 (±1.13)*   |
| Muscle layer (mm) ..............   | 150             | 0.176 (±0.08)*  | 0.197 (±0.01)*   |
| Circumference (mm) ............   | 10              | 5.293 (±0.63)*  | 4.060 (±0.11)*   |
| Chicken:                         |                 |                 |                 |
| Villus height (mm) ...........    | 150             | 5.264 (±0.70)*  | 5.260 (±0.78)*   |
| Muscle layer (mm) ..............   | 150             | 0.875 (±0.15)*  | 0.722 (±0.13)*   |
| Circumference (mm) ............   | 10              | 8.889 (±0.11)*  | 10.807 (±0.17)*  |
| Mouse:                           |                 |                 |                 |
| Villus height (mm) ...........    | 150             | 6.181 (±0.72)*  | 6.005 (±0.67)*   |
| Muscle layer (mm) ..............   | 150             | 0.278 (±0.01)*  | 0.309 (±0.01)*   |
| Circumference (mm) ............   | 10              | 9.572 (±0.26)*  | 8.391 (±0.26)*   |
| Note: \( N = \) sample size. Values denoted by different superscripted letters differ significantly at \( P < 0.05 \).
smooth muscle cells (Fig. 3A). In the anterior portion of the small intestine, there are only a few goblet cells, and the enterocytes have no vesicles as found in the sunbird. The lamina propria mucosae is solid and contains aggregations of lymphatic cells. Eversion of the intestine caused the villi to broaden at the base and to become somewhat notched/Indented along their surface (Fig. 3B), but it did not affect the structural integrity of the mucosal epithelium or the enterocytes. We measured a significant decrease of villus height and an increase of the thickness of the muscle layer. Circumference of the gut decreased after eversion but increased again when everted and incubated (Table 1). The surface magnification of the gut increased significantly after eversion and decreased following eversion and incubation (Table 1). Thus, a decrease of villus length goes along with a decrease of circumference and an increase of muscle layer thickness. Clearly, this is a consequence of mechanical stretching of the villi at their base. The decrease in villus length and surface magnification after eversion and incubation apparently depends on stretching the intestine and compressing the villi. However, under light-microscopical investigation, the tip of the villi appear intact. In our morphometric analysis, which was based on three individuals only, individual and tissue handling had significant effects on villus length ($F_{3,11} = 661.2$, $P < 0.0001$), circumference ($F_{3,11} = 8.8$, $P = 0.0004$), and surface magnification ($F_{3,11} = 14.5$, $P < 0.0001$). Individuals had no significant effect on the thickness of muscle layer. The mean squares of individuals for these parameters showed that one mouse (M1) had very low values in all morphometric parameters.

Discussion

The results of our comparisons are straightforward. We observed heavy damage to the histology and cytology of the intestinal tissues of sunbird together with associated changes in morphometry. In this species, the intestinal villi are long and thin, and the intestinal mucosal epithelium is delicate. The shaving of the villi by the everted sleeves method coincides with the low measurements of uptake rates. The same may hold for growing birds (Turdus philomelos; Konarzewski and Starck 2000). In chicken and mice, the intestinal tissue is more robust against mechanical impact. Throughout the procedure it maintained its normal structure, although height of villi and surface magnification were affected by mechanical stretching during eversion. These morphometric parameters were not associated with tissue damage. Other changes that could affect uptake capacity but that we did not measure include changes in microvillus ultrastructure, membrane-transporter density, cellular energy stores supporting active transport, and paracellular barrier integrity (possibly affected by stretching the tissue and compressing the villi, or incubation solution chemical effects). Considering our findings of histological changes during the procedure, and many other unknowns, we urge caution in

Figure 2. Chicken. A. Histology of the small intestine, anterior portion, untreated. B. Histology of the anterior portion of the small intestine after eversion. C. After eversion, 5-min preincubation, and 1-min incubation in a stirred medium. Scale bar = 500 µm.

morphometric changes were not associated with histological and cytological damage as described for the sunbird.

Although mammalian and avian intestines differ in several details of their microscopic anatomy, the effects of tissue handling on mouse intestinal tissue were similar to those described for chicken. The noneverted mouse intestine has long and broad villi with a solid core of connective tissue and stripes of
In conclusion, our study has shown that intestinal tissue of different species of birds (and mammals) reacts differently to tissue handling during the everted sleeves method. The method may have detrimental effects on intestinal mucosal epithelium when tissue is delicate, e.g., in sunbirds and possibly also in young birds of other species. Tissue damage may be so extreme that results of uptake measurements become unreliable. Consequently, we strongly suggest that the impact of the everted sleeves method on tissues has to be evaluated histologically for any species before reliable results can be published. This concern not only new species but also such species that have already been examined but for which no histological checks have been made.

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Literature Cited


