A simple method for measuring intestinal solute uptake in vitro

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Summary. We describe a method of measuring intestinal solute uptake that combines the virtues of simplicity, good tissue viability as reflected in high uptake rates, and reduction of unstirred layers comparable to the best reported for chambers. An everted sleeve of intestine is mounted on a grooved rod stationed immediately over a stirring bar rotating at 1,200 rpm. The effect of edge damage is negligible. The coefficient of variation of uptake measurements is 7%. For mouse intestine and D-glucose at high concentrations an incubation time of 1 min represents a suitable compromise among several competing criteria. L-glucose is used to correct active D-glucose uptake simultaneously for adherent fluid and for passive uptake. The technique's general utility is illustrated by its application to intestines of four species representing four classes of vertebrates.

Introduction
This paper introduces a simple and effective technique for measuring solute uptake in vitro, using everted sleeves of intestine mounted on rods. In the years since introduction of the everted sac technique revolutionized the study of intestinal absorption, it has become appreciated that the type of preparation used to measure uptake profoundly affects measured values of kinetic constants for active transport (Thomson and Dietschy 1980a). The variety of techniques in use at present makes comparison of kinetic data from different laboratories difficult. Thus, our goal was to develop a method applicable to intestines of even the smallest vertebrates, as the basis for a broadly based comparative study of intestinal absorption.

Our method incorporates advances made in measuring uptake in chambers (Schultz et al. 1967; Lukie et al. 1974) and in controlling unstirred layers (Westergaard and Dietschy 1974). It resembles a preparation developed by Malathi et al. (1973), from which it differs by mounting the sleeve on a grooved rod rather than polyethylene tubing and by improved stirring. Compared to intestinal ring preparations (Crane and Mandlebaum 1966, Miller et al. 1974), our sleeve technique shares their simplicity but has two advantages: isolation of the serosal surface from the incubation medium, so that one measures only uptake across the apical membrane rather than across both cell faces simultaneously; and the feasibility of using much higher stirring rates and thereby reducing the unstirred layer at the apical membrane. Chamber techniques offer the same two advantages, but the sleeve technique involves simpler equipment and simpler tissue preparation and mounting, possibly resulting in less edge damage and better viability. In practice, the sleeve technique proves to yield higher uptake rates than the chamber technique. Furthermore, the sleeve method can be applied to small animal species whose narrow intestinal diameter makes it difficult to obtain enough tissue to mount in a chamber.

We describe experimental procedures and then validation experiments to select appropriate rinse and incubation times, to separate active from passive uptake, and to determine reproducibility. Our use of L-glucose uptake simultaneously to correct for adherent fluid and for passive uptake is applicable to other uptake measurement procedures besides the sleeve method. Finally, we report glucose and proline uptake measurements in 4 species, to illustrate the technique's usefulness in comparative studies.
Materials and methods

Tissue preparation and mounting. We used mouse intestine for experiments to develop and validate the technique. Male white Swiss Webster mice weighing 30–40 g were anesthetized with 0.07 ml Diabital (sodium pentobarbital). A catheter was inserted just distal to the pyloric sphincter, the intestine cut just proximal to the cecum, and the entire small intestine then perfused with cold (–2 °C) mammalian Ringer. Solution composition (in mM) was 128 NaCl, 4.7 KCl, 2.5 CaCl₂, 1.2 KH₂PO₄, 1.2 MgSO₄, 20 NaHCO₃, pH 7.3–7.4 at 37 °C, 290 mOsm. Solutions containing glucose or proline were prepared by isosmotic replacement of NaCl. The intestine was quickly excised, rinsed in cold Ringer, and everted with a glass rod. After the intestine’s length had been measured, pieces 1.5 cm in length were cut from the jejunum, and each piece was slipped over the tip of a glass rod 4 mm in diameter and 20 cm long. (We used rods 2–8 mm in diameter to measure uptake by intestines of larger or smaller species.) Each rod had one groove 1 mm from its tip and another groove 11 mm from the tip. The sleeve of tissue was secured to the rod at one groove with surgical thread, was stretched and allowed to spring back, and was then secured at the second groove. Excess tissue outside the 1-cm length between the two grooves was cut away. The tissue was then gently rinsed by a jet of cold Ringer.

During this preparatory phase mounted and unmounted tissues were kept in cold Ringer gassed with 5% CO₂–95% O₂. About 1/2 h elapsed from anesthetization of the animal to mounting of the first sleeve, and 10–12 sleeves from 1–4 animals could be mounted within the next 1/2 h. We found that d-glucose uptake declined between 1/2 h and 1 h after anesthetization but thereafter remained constant at least 2 h. We therefore made uptake measurements between 1 and 2 h after anesthetization.

Flux determinations. Probe molecules used were 1–3H d-glucose, 6–3H d-glucose, 14C(U) d-glucose (U = uniformly labelled at all carbons), 3–3H L-proline, 14C(U) L-proline, 1–3H l-glucose, and 1–14C dodecanol (lauryl alcohol). Marker compounds used were 1–3H PEG or 1–14C PEG to measure adherent fluid (PEG = polyethylene glycol, MW 6000), 1–14C or 3–3H l-glucose to measure adherent fluid and passive uptake simulta- neously. 14C dodecanol and 14C(U) l-proline were obtained from ICN Pharmaceuticals, other tracers from New England Nuclear.

Just prior to a flux measurement a rod with mounted sleeve of intestine was precubed with 5 min at 37 °C in Ringer gassed with 5% CO₂–95% O₂. The rod was then transferred to a flat-bottomed water-jacketed test tube 18 mm in diameter and containing 8 ml oxygenated Ringer with probe and marker solutes at 37 °C. The tube contained a 15-mm spin bar rotated at 1,200 rpm (range 1,160–1,240 rpm, checked with a stroboscope) and the rod was held vertically a few mm over the spin bar. After up to 8 min the incubation was terminated by removing the rod and quickly rinsing it in a 30-ml beaker of cold Ringer stirred at 1,200 rpm for 20 s. This rinse time was chosen to wash away most of the adherent fluid but little of the absorbed probe (see Results). Finally, the flat end of the rod was drained by touching it to a filter paper, and the mounted tissue was cut from between two grooves with a razor blade and placed in aared glass scintillation vial. An incubation bath sample was taken before incubation of the tissue for subsequent counting. Bath glucose or proline activity dropped during tissue incubation by only a few percent at low probe concentrations, and no drop was detectable at higher concentrations. Morphological examination showed that the villi are still intact at 8 min.

When 14C dodecanol at tracer concentrations was added to Ringer at 37 °C, it rarely all dissolved, and crystals could be seen on the surface of the solution. Solution counts/min dropped rapidly with time and then levelled off. By adding tracer dodecanol to Ringer 3–4 h before an experiment and filtering through No. 1 filter paper, we obtained a solution whose count/min remained constant with time when stirred at 37 °C. Since bath dodecanol activity decreased by about 10% during tissue incubations due to tissue uptake of dodecanol, bath samples were taken before and after the incubation, and the mean bath activity was used in subsequent calculations.

Counting methods. In some cases, tissue was prepared for counting by drying overnight in an oven at 70 °C. After the tared vial with the tissue had been weighed to determine tissue dry weight, 0.1 ml distilled water was added to moisten the tissue, then 1 ml TS-1 tissue solubilizer (Research Products International) was added. Vials were incubated at 55 °C overnight, and 10 ml Econofluor (New England Nuclear) was added after cooling. In other cases (to be discussed), and in experiments with tracer dodecanol, overnight drying and incubation were omitted; instead, tissue wet weight was determined, solubilizer was added, and vials were incubated for 3 h before adding Econofluor. This procedure resulted in no loss of labelled dodecanol from spectrometer.

Radioactivity was determined on a Beckman LS 235 liquid scintillation spectrometer with automatic external standardization (AES) and windows set as follows: 3H channel 0–120 (0–0.24 megavolts), 14C channel 350–1,000 (0.7–2.0 megavolts). Quenched standards were included in every group of samples. Efficiency of counting 14C and 3H was plotted against AES value, and calibration curves for 14C, 3H, and 14C spillover into the 3H channel were obtained by determining the unique fourth-order polynomial that passed through the standard points. All solution and tissue samples were corrected for variable quenching to obtain disintegrations per minute. Typical counting efficiencies and 14C spillover were (3H, 14C, 14C spillover): bathing solutions 24%, 40%, 5%; tissue samples, 20%, 35%, 8%.

Washout experiments. Experiments to determine rate of washout of tracer from an incubated tissue were performed in the same manner as flux determinations except that, after incubation, the tissue was rinsed in a series of beakers of 25 ml cold Ringer without tracer for periods consecutively increasing from 2 s to 5 min. A 2-ml aliquot of each rinse solution was added to 10 ml PCS (Amersham) and counted, and the total DPM washed from the tissue were calculated. At the end of the rinse the tissue was counted as described under flux determinations (except in PCS instead of Econofluor), and DPM remaining in the tissue were calculated.

Calculations. Intestinal uptakes and fluxes in the literature have been standardized to various measures of the quantity of tissue, without agreement having been reached on the best measure. We routinely standardize to four measures: length, dry weight, nominal surface area, and villous surface area. In this paper, we report uptakes in mouse intestine with respect to length and dry weight; the other two measures yield essentially the same conclusions for the purposes of this paper.

Solute "spaces" were calculated as M/C, where M = DPM of marker or probe associated with the tissue sample, C = DPM of marker associated with the sample, R = (DPM
of probe/DPM of marker) in the incubation solution, \( H = \) (DPM of probe/nmole of probe) in the incubation solution, \( t = \) incubation time, and \( m = \) dry weight or length of intestine. \( J \) has the units of nmole/min·cm length or else nmole/min·mg dry weight depending on the units of \( m \).

Diffusion coefficients \( D \) were estimated as \((182.2/MW)^{1/3} \times 9.29 \times 10^{-6} \text{ cm}^2/\text{s}\), taking \( D \) of mannitol (MW 182.2) as 37°C as \( 9.29 \times 10^{-6} \text{ cm}^2/\text{s} \) (Sallee and Dietzchy 1973).

Results are given as the mean ± SEM (\( n = \) sample size). The \( t \)-test was used for tests of significance at the \( P < 0.05 \) level.

Results

Isolation of the serosal and mucosal compartments

The intestinal sleeve is secured to the rod by thread tied over two grooves. We wish to measure tissue uptake from the mucosal solution across the apical cell surface. Could some isotope instead be taken up across the serosal face, having passed from the mucosal solution around the tissue at the seeping threads into the serosal layer of fluid? This possibility ("edge damage") was tested by using rods identical to the usual solid rods except for being hollow and having perforations between the two grooves (but still sealed at the tip). After the tissue sleeve had been secured on the rod, 200 μl Ringer was placed in the serosal compartment inside the rod, and the tissue was incubated for 4 min in Ringer with \(^3\)H PEG. After a 4-min incubation and 20-s rinse of the mucosal surface (see next section for selection of these times), \(^3\)H PEG activity (DPM/μl) in the serosal fluid was only 0.12 ± 0.03% (\( n = 6 \)) of that in the mucosal fluid, and the total amount of \(^3\)H PEG in the serosal fluid was only 3.3 ± 1.0% (\( n = 6 \)) of that adhering to the tissue. These amounts are negligible, and the serosal surface may be considered isolated from the mucosal surface by the tissue.

Choice of incubation and rinse times

The selection of these times involves a compromise among several considerations. The post-incubation rinse should be long enough to wash off most of the adherent fluid and brief enough to wash out little solute taken up into the cells. The incubation time should be long enough to permit complete equilibration of adherent fluid with bathing solution, long enough that uptake is appreciable and accurately measurable, and short enough that isotope taken up is still largely in the tissue and that little has reached the serosal surface. Incubation should be terminated in the linear uptake vs-time phase; this requires a time brief enough that backflux from cells to mucosal bathing solution is still negligible, yet long enough that the effect of the initial lag for equilibration of the adherent fluid is negligible and the uptake vs-time curve extrapolates close to the origin. We resolved these compromises on the basis of the following measurements.

To select the post-incubation rinse time, we performed three experiments in which we incubated tissues for 8 min in Ringer with \(^1\)H D-glucose and the adherent marker \(^14\)C PEG, then rinsed in a series of 75-ml beakers without isotope, dried, and counted. The percentage of the \(^14\)C PEG (upper curve) or \(^1\)H D-glucose (middle curve) present in the tissue at \( t = 0 \) remaining after various times is plotted against time. The lower curve is the efflux time constant (%/min) calculated for D-glucose from the middle curve. Values plotted are the mean ± SEM for three experiments. Error bars for most values of the upper curve are not depicted because they would be smaller than the plotted points. Note that PEG washes out much more rapidly than does D-glucose, and that the D-glucose efflux constant declines to an asymptote (representing efflux of intracellular glucose) within a few minutes.

Fig. 1. Sleeves were incubated for 8 min with \(^1\)H D-glucose and \(^14\)C PEG, then rinsed in a series of 75-ml beakers without isotope, dried, and counted. The percentage of the \(^14\)C PEG (upper curve) or \(^1\)H D-glucose (middle curve) present in the tissue at \( t = 0 \) remaining after various times is plotted against time. The lower curve is the efflux time constant (%/min) calculated for D-glucose from the middle curve. Values plotted are the mean ± SEM for three experiments. Error bars for most values of the upper curve are not depicted because they would be smaller than the plotted points. Note that PEG washes out much more rapidly than does D-glucose, and that the D-glucose efflux constant declines to an asymptote (representing efflux of intracellular glucose) within a few minutes.
Fig. 2A–C. Tissue uptake of PEG (A), D-glucose (B), and L-proline (C) as a function of duration of incubation. A PEG uptake expressed as the PEG space (μl) per cm length of intestine. B: total uptake of 1-3H D-glucose at 1 mM, corrected for adherent fluid on the basis of the 14C PEG space, and expressed as the uptake at the given time divided by the uptake at 4 min measured in an adjacent sleeve from the same animal. •: active uptake of 14C D-glucose at 50 mM, corrected for adherent fluid and passive uptake on the basis of the 1-3H L-glucose space, and normalized to the uptake at 3 min measured in an adjacent sleeve from the same animal. Dashed lines in B connect the points at 4 min (o) or 1 min (•) with the origin. C: total uptake of 14C L-proline at 50 mM, corrected for adherent fluid on the basis of the 3H PEG space and normalized to 1 cm length of intestine. Note that the adherent fluid equilibrates with PEG in about 2 min (A), that D-glucose uptake at low concentrations is linear with time for at least 8 min (B), and that D-glucose (B) and L-proline (C) uptake at 50 mM becomes nonlinear with time in less than 4 min.

for the 1st min is dominated by a fast component paralleling the washout of PEG and presumably representing glucose in the adherent fluid. This rapid phase is followed by a slow glucose efflux component presumably representing intracellular glucose. From the rate constant of this slow phase, 2.8 ± 0.1%/min (n = 3), one calculates that only about 1% of the intracellular glucose is lost in a 20-s rinse that yields a PEG space (adherent fluid space) of 5.6 ± 0.6 μl/cm length or 0.81 ± 0.08 μl/mg dry weight (n = 18). This PEG space proves to be a small correction to measured total glucose and proline uptakes at 2 min. At 0.5 mM only 6% of the total tissue D-glucose or 15% of the total tissue proline is extracellular, and the correction is still only 10% at 50 mM D-glucose and 25% at 50 mM L-proline.

Our procedure does overestimate this small correction because the diffusion coefficient of glucose and proline exceeds that of PEG. While the incubation period (see next four paragraphs) generally permits complete equilibration of tracer D-glucose or proline and PEG between the bath and fluid, more tracer D-glucose or proline than PEG leaves the adherent fluid again during the 20-s rinse, so that the estimate of adherent glucose or proline is an overestimate. In practice, this error did not arise in our studies of D-glucose active transport: as will be described, we used L-glucose rather than PEG to correct simultaneously for adherent fluid and passive uptake. L-glucose and D-glucose have the same diffusion coefficient.

To select the incubation time, tissues were incubated for various times in Ringer containing tracer glucose, L-proline, and PEG, and tissue uptake was determined after a 20-s rinse (Fig. 2). Figure 2A shows that tissue PEG reaches an asymptote by 2 min and reaches half this value at about 1/2 min. From their diffusion coefficients relative to that of PEG, one estimates this half-time at about 11 s for D-glucose and 9 s for L-proline. Thus, the effect of the lag for equilibration of the unstirred layer with glucose or proline should be slight in an uptake experiment lasting several minutes; the uptake-vs-time line for these solutes should extrapolate close to the origin.

Figure 2B indicates that D-glucose uptake at low concentrations (1 mM) is in fact approximately linear with time to at least 8 min. As a quantitative test, we compared uptake from solutions containing 1 mM 1-3H D-glucose in adjacent pieces of intestine from the same animal, one piece used as control and incubated for 4 min, the adjacent piece incubated for 1, 2, 4, or 8 min. This procedure reduced the effect of individual variation among animals. As illustrated in Fig. 2B, D-glucose uptake at 2 min was within 6% of the value predicted by assuming linear uptake between 0 and 4 min extrapolating through the origin. This result was obtained whether uptake was normalized to sleeve length or to dry weight.

At high concentrations, however, uptake of D-glucose or L-proline becomes non-linear with time in less than 4 min. At 50 mM, for example, D-glucose active uptake is linear for only about 1.5 min.
(Fig. 2B), l-proline uptake for somewhat over 2 min (Fig. 2C). Hence uptake rates calculated from 2-min incubations underestimate those calculated from 1-min incubations by 15% for 50 mM d-glucose, by 8% for 50 mM l-proline. One might therefore conclude that a 1-min incubation is preferable to a 2 min incubation at 50 mM, and this reasoning is correct for d-glucose. However, a 1-min incubation has a significant offsetting disadvantage for l-proline: PEG equilibration with the unstirred layer is not yet complete at 1 min, tending to underestimate the adherent fluid correction, which is about 25% of total uptake for l-proline at 50 mM. (This consideration is irrelevant for d-glucose, for which we base the adherent fluid correction on l-glucose, which has an identical diffusion coefficient.) Thus, a 2-min incubation may be the best compromise for l-proline at 50 mM, while 1 min is preferable for d-glucose at 50 mM. Incubations can be safely extended to 4 min at low concentrations. In short, for accurate quantitative work the optimal choice of incubation time may vary with solute and even with concentration.

Figure 2B also suggests that little d-glucose taken up escapes to the serosal surface during short incubations, as such loss would make the uptake vs-time curve non-linear. This condition was checked directly by mounting mouse intestine slices on the hollow glass rods described in the section 'Isolation of the serosal and mucosal compartments' and incubating in Ringer containing 0.5 mM 1-3H d-glucose. After 4 min the quantity of 3H d-glucose in the serosal fluid was only 5.7 ± 1.6% (n = 7) of that in the tissue. We performed the same experiment with similar results in kangaroo rat jejunum, which has one of the highest D-glucose uptake rates of the species that we have studied, and in desert iguana ileum, which is particularly thin: serosal 3H D-glucose respectively 8.4 ± 1.5% (n = 6) and 5.1 ± 1.7% (n = 2) of tissue 3H D-glucose after 4 min.

Hence for d-glucose we chose a 1 min incubation time as a compromise yielding negligible loss of glucose to the serosal fluid, equilibration of the adherent fluid, uptake in the linear phase, and appreciable amounts of uptake.

Reproducibility

To assess reproducibility, we compared glucose uptake rates in adjacent pieces of jejunum from the same mouse, and then compared the mean of the two values for different individual animals. These comparisons were made for 4–10 animals on each of five different diets. For example, for seven animals on a laboratory chow diet the coefficient of variation (C.V.) was 7 ± 2% (n = 7) within an animal and 19% between animals. In the five series the mean C.V.'s within an animal ranged from 5 to 9% and averaged 7%, while the C.V.'s between animals ranged from 9 to 46% and averaged 27%. Thus, as expected, agreement is closer for uptake rates in adjacent tissues of the same animal than for uptake rates measured in different animals. Hence most of our results are based on using one piece of tissue as control for an adjacent piece (e.g., Figs. 2, 3, and 5).

Concentration dependence of active transport

D-glucose was accumulated by mouse intestinal sleeves to a much higher concentration than in the mucosal bathing solution. For example, with 1 mM d-glucose in the bath the tissue uptake rose from 4.3 ± 0.7 (n = 4) nmoles/mg dry weight at 1 min to 34 ± 8 (n = 3) nmoles/mg at 8 min. Since tissue water content (total water – PEG space) was 4.3 ± 0.4 (n = 8) µl/mg, glucose concentration related to tissue water at 8 min was 34/4.3 = 7.9 mM, 7.9 times the bath concentration. However, much tissue water is not intracellular water of the epithelial cells. The scraped mucosa represents only 68% of the dry weight of the intestine, and some of that 68% is muscularis mucosa. Furthermore, some tissue water associated with the mucosa is inaccessible to PEG (e.g., water in the lateral spaces). Thus, the estimated cell-to-bath concentration ratio of 7.9 at 8 min underestimates the true value, probably by at least a factor of 2.

In contrast, l-glucose was not taken up against a concentration gradient by mouse intestinal sleeves, in agreement with results from other methods and other species. After 4 min incubation in tracer concentrations of 3H l-glucose, the tissue concentration (corrected for adherent l-glucose by means of 14C PEG) was only 8 ± 0.8% (n = 9) of the bath concentration, whereas the figure for d-glucose at 4 min was 837 ± 93% (n = 8, bath concentration 0.5 mM). This low rate of passive l-glucose uptake down its concentration gradient is reflected in the fact that the l-glucose space somewhat exceeds the PEG space at 4 min, by 1.52 ± 0.06 (n = 10) times. Destruction of the membrane barrier by preincubation for 20 min with Triton X at 0.1–1 mg/ml (Eckert and Murakami 1972) increased both the l-glucose space (by 170%) and the PEG space, but they became equal: their ratio dropped from 1.52 to 1.07 ± 0.05 (n = 3).

Intestinal uptake of d-glucose involves a passive component as well as an active component.
Thomson and Dietschy (1980b) have emphasized the possibility of significant errors in calculating kinetic parameters of active transport from measured uptake rates if one does not subtract the passive component. They made this correction by using $^3$H dextran (as an adherent fluid marker) and $^{14}$C D-glucose to measure total uptake in one tissue, then using $^3$H dextran and $^{14}$C L-glucose to measure passive uptake in another tissue. The rate of passive uptake is assumed equal for L-glucose and D-glucose. We simplified this procedure by routinely using D-glucose at various concentrations ($^{14}$C label) and L-glucose at trace concentrations ($^3$H label) in the same tissue. L-glucose uptake at the concentration of D-glucose was calculated on the assumption that the uptake-vs-concentration relation for L-glucose is linear. Subtraction of this L-glucose uptake from D-glucose uptake corrects simultaneously for adherent fluid and passive uptake, yielding active uptake directly. This procedure has the further advantage that D-glucose and L-glucose have the same diffusion coefficient, hence the correction for adherent fluid using L-glucose is not subject to the previously discussed error arising from unequal diffusion coefficients of D-glucose and PEG.

To investigate the kinetics of active transport, we incubated 3–5 adjacent pieces of jejunum in solutions of varying D-glucose concentration labelled with $^{14}$C D-glucose and $^3$H L-glucose. As depicted in Fig. 3, active uptake displays the anticipated hyperbolic relationship to D-glucose concentration. Fitting these data to the Michaelis-Menten equation by the Gauss-Newton method of nonlinear least-squares regression as described by Duggleby (1981) yields $J_{\text{max}} = 932$ nmoles/min·cm and $K_m = 6.2$ mM for pooled data from all animals (Fig. 3). When $J_{\text{max}}$ and $K_m$ were instead calculated separately for each individual animal, the estimates were similar: $J_{\text{max}} = 927 \pm 58$ nmoles/min·cm ($n = 8$); $K_m = 6.2 \pm 0.3$ mM ($n = 8$).

The L-glucose uptake may be used to estimate $P_{\text{glucose}}$, the passive permeability coefficient of D-glucose or L-glucose. Calculated as $(\text{DPM uptake/min·mg})/(\text{DPM/µl bath})$, $P_{\text{glucose}}$ averaged $0.75 \pm 0.008$ µl/min·mg ($n = 10$) or $0.62 \pm 0.08$ µl/min·cm ($n = 10$). Figure 3 illustrates that the passive uptake component is only a small fraction of total D-glucose uptake: about 1% at 1 mM and 4% at 50 mM.

**Effects of unstirred layers**

Unstirred layers can yield enormous errors in intestinal uptake studies on both actively and passively absorbed solutes, by permitting solute concentration at the apical membrane to drop below solute concentration in the well-stirred bathing solution and hence causing the uptake rate for that bathing concentration to be underestimated (Dietschy et al. 1971; Winne 1973; Westergaard and Dietschy 1974). The magnitude of the error increases with permeability for a passively absorbed solute, and with $J_{\text{max}}$ decreasing concentration, and decreasing $K_m$ for an actively absorbed solute. $K_m$ is there by overestimated, but $J_{\text{max}}$ is correctly estimated even in the presence of an unstirred layer if uptake measurements are extended to sufficiently high solute concentrations. We used two methods to check the significance of unstirred-layer effects in mouse intestinal sleeves.

First, we measured D-glucose uptake from 1 mM solutions as a function of stirring rate. Uptake increased 2-fold as stirring rate increased from 200 to 1,200 rpm (Fig. 4). As predicted theoretically (Winne 1973; Thomson and Dietschy 1977), the effect of stirring decreased with increasing D-glucose concentration (Table 1): a change in stirring rate from 200 to 1,200 rpm increased uptake 3.9 times at 0.0185 mM but only 1.1 times at 50 mM. The explanation is that the unstirred layer and the membrane constitute two series resistances to glucose uptake. As glucose concentration increases, the glucose carrier becomes saturated, membrane resistance to glucose thereby increases, and the relative importance of the unstirred layer decreases. To minimize unstirred-layer effects, we
made all other measurements of solute uptake reported in this paper at 1,200 rpm, the highest stirring rate obtainable with our equipment.

Second, we measured the uptake of dodecanol (lauryl alcohol), a very lipid-soluble molecule whose membrane permeability is so high that its intestinal uptake is limited by the diffusional resistance of the unstirred layer (Westergaard and Diettsch 1974). As Fig. 5 shows, uptake of dodecanol at a stirring rate of 1,200 rpm is so rapid that a plateau value is reached by 2 min. Decreasing the stirring rate from 1,200 to 200 rpm decreases uptake at 4 min by a factor of 3.9, from $571 \pm 66 \ (n=16)$ to $145 \pm 5 \ (n=7) \ \mu l/cm$. Since the 4-min value is a plateau value at 1,200 rpm, this factor greatly underestimates the effect of stirring on the dodecanol uptake rate. If one makes a crude estimate of the dodecanol uptake rate at 1,200 rpm by assuming the uptake value at 30 s to fall within the linear phase, one obtains a flux/concentration ratio of $296 \pm 32 \ (n=5) \ \mu l/min \cdot cm length$. This estimate will be used in the Discussion to compare stirring efficiency in chambers and in sleeves.

After correction for dodecanol and water in the PEG space, the plateau value of dodecanol uptake at 1,200 rpm and 4 min corresponds to a dodecanol concentration in tissue water $25 \pm 4 \ (n=3)$ times that in the bathing solution. This steep concentration ratio is not due to active transport but to rapid esterification of dodecanol by the intestine and partition into intestinal lipid stores (Hanson and Mead 1965).

### Tissue metabolism of absorbed solute

Suppose that the intestine metabolizes absorbed solute. This might cause the measured uptake rate to underestimate the true value—but only if metabolism yielded a volatile product (e.g., $^{14}CO_2$ or $^3H_2O$) that escaped from the tissue before it was counted.

To assess this potential source of error, we incubated tissues in 50 mM D-glucose solutions containing two tracers: $1^3H$ D-glucose and $^{14}C(U)$ D-glucose. After 4 min tissues were rinsed, blotted, cut in half, and weighed. One half was immediately prepared for counting in a closed scintillation vial, while the other half was dried overnight in an oven before closing the vial. The $^3H$ and $^{14}C$ spaces in wet tissue ($\mu l/mg$ wet tissue) proved to be the same: $^3H / ^{14}C = 0.96 \pm 0.03 \ (n=8)$; probability that the value does not differ from 1 is $P > 0.2$.
by a paired t-test. In dry tissue the $^{3}$H space ($\mu$l/mg dry tissue) was significantly less than the $^{14}$C space: $^{3}$H/$^{14}$C = 0.61 ± 0.01 ($n = 4$), $P < 0.001$. This effect of drying was entirely on the $^{3}$H space; it was 33 ± 2% ($n = 9$) lower in dried than in wet tissues ($P < 0.001$). Drying had no effect on the $^{14}$C space: ratio for dried to wet tissue 1.03 ± 0.03 ($n = 7$), $P > 0.2$. Thus, mouse intestine metabolizes some absorbed $^{3}$H D-glucose (and also 6-$^{3}$H D-glucose in other experiments) to a volatile compound (presumably $^{3}$H$_{2}$O) that escapes during drying. Our experiment would not detect loss of label during the incubation itself, prior to sealing the wet tissue in a vial for counting. However, this loss is probably slight. For instance, the fraction of transported $^{14}$C glucose oxidized to $^{14}$CO$_{2}$ is less than 3% for 4-min incubations of rat jejunum (Wilson and Dietschy 1974) and less than 11% even for 1-h incubations of hamster intestine (Landau and Wilson 1959).

The significance of these observations is that incubation of mouse intestine with $^{3}$H D-glucose followed by drying yields an underestimate of the D-glucose uptake rate by about 1/3. This is the procedure that we used in the experiments of Figs. 1 and 4 and Table 1, carried out before we had discovered the conditions under which isotope escape due to metabolism occurred. Reconsideration of these experiments indicates that their qualitative conclusions are still valid, although their quantitative values for glucose uptake are somewhat too low.

A similar series of experiments with L-proline uptake in mouse intestine yielded the following results ($n = 4$ in each case): $^{3}$H space/$^{14}$C space = 1.11 ± 0.06 in wet tissues, 1.02 ± 0.09 in dried tissues; space in dried tissues / space in wet tissues = 0.97 ± 0.02 for $^{14}$C, 0.89 ± 0.04 for $^{3}$H. None of these values differs significantly from unity by a paired t-test, suggesting that isotope loss via a volatile metabolite is not significant for proline.

Discussion

We consider four issues: validation of the new method; comparison of it with other methods; effects of unstirred layers; and application to other vertebrate species.

Validation of the method

We have shown that movement of PEG across the intestinal sleeve is negligible. This means that edge damage is negligible, and that the serosal surface and mucosal solution are effectively isolated by the tissue.

The rinse time (20 s) and incubation time (1 min) chosen for D-glucose sufficed to minimize the adherent-fluid correction, minimize the loss of D-glucose to the serosal surface during the incubation period, minimize back-flux from cells to mucosal bath, allow time for equilibration of bath glucose with the unstirred layer, and thereby ensure that uptake was in the linear phase (with time) but still readily measurable. Naturally, the times that we found suitable for D-glucose may not be suitable for other solute. Uptake of L-proline is approximately linear with time for up to 2 min (Fig. 2C), whereas the linear phase of uptake for dodecanol is less than 2 min (Fig. 5). However, for two reasons we think that incubation times of 1 or 2 min at high solute concentrations, and up to 4 or 8 min at low or tracer concentrations, may also prove appropriate for D-glucose in other vertebrate species. First, serosal loss of D-glucose at 4 min is negligible in mouse, kangaroo rat, and desert iguana intestines, although the intestine in the latter two species is as thin as or thinner than that in mouse, and although only one other species among the 18 that we have studied has as high glucose transport rates as these three species. Thus, serosal efflux at 1 min will probably be even lower in species with lower uptake rates. Second, the passive permeability of glucose in intestines of the other vertebrate species studied is similar to or lower than that in mouse intestine. Thus, back-flux from cells to mucosal bath at 1 min in these other species should be no more serious than in mouse intestine. For these two reasons D-glucose uptake in other vertebrate species besides mouse might still be in the linear phase at 1 min. In agreement with this conclusion, Thomson and Dietschy (1980b) found glucose uptake to be linear with time for at least 8 min in rabbit intestine.

Tissue metabolism introduces an error when D-glucose uptake in mouse intestine is measured with $^{3}$H D-glucose and the tissue is dried prior to counting. This error can be eliminated either by using $^{14}$C D-glucose, or else by not drying the tissue. Similarly, Ferraris (1982) found that up to 60% of the $^{3}$H from $^{3}$H D-glucose absorbed by surgeonfish intestine became volatile. We have preliminary evidence for significant loss of $^{3}$H D-glucose in sturgeon, two species of frugivorous bats, and two species of herbivorous lizards when tissues are dried. Clearly, this possibility needs checking for each species and solute studied. When in doubt, we recommend using $^{14}$C D-glucose and not drying tissues.
Table 2. Comparison of everted intestinal sleeves with other intestinal preparations in glucose or proline uptake rates. Uptake rates are given as means ± SEM (n). Sleeve uptake rates are from this paper.

<table>
<thead>
<tr>
<th>Species</th>
<th>Solute</th>
<th>Concentration (mM)</th>
<th>Uptake rate (nmoles/min·mg)</th>
<th>Which other method?</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>sleeve</td>
<td></td>
</tr>
<tr>
<td>Rat</td>
<td>D-glucose</td>
<td>1.0</td>
<td>0.6 ± 0.1 (6)</td>
<td>Chambers*</td>
</tr>
<tr>
<td>Mouse</td>
<td>L-proline</td>
<td>0.5–0.6</td>
<td>3.5 ± 0.8 (10)</td>
<td>Rings†</td>
</tr>
<tr>
<td>Mouse</td>
<td>Monosaccharide</td>
<td>0.5–0.6</td>
<td>9.3 ± 0.8 (5)</td>
<td>Rings‡</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.28</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.48 ± 0.09 (5)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.70 ± 0.02 (5)</td>
<td></td>
</tr>
</tbody>
</table>

* Thomson et al. (1982) reported active plus passive uptake as 0.40 ± 0.08 nmoles/min·mg, of which 70% (hence 0.28) was active.
† Our sleeve value is of the active component
‡ Lerner and Kratzer (1976) measured active uptake from 0.6 mM solutions. Our sleeve value is for active plus passive uptake from 0.5 mM solutions, but passive uptake is less than 10% of the total at this concentration (Lerner and Kratzer 1976)
§ Calingaert and Zorzoli (1965) reported active plus passive uptake of 6-deoxy-D-glucose from 0.6 mM solutions, in units of μmole/30 min·mg wet weight. We converted this value to a dry weight basis (dry weight of a mouse intestine reported as 19% of wet weight by Calingaert and Zorzoli). Our sleeve value is of active plus passive uptake of D-glucose from 0.5 mM solutions (14C label). In rat intestine the 6-deoxy-D-glucose uptake at this concentration is half that of D-glucose (Syme and Levin 1980)

Comparison with other methods

The reproducibility of the everted sleeve technique (mean coefficient of variation for D-glucose uptake, 7%) is similar to that of the intestinal ring method (C.V. 5%; Crane and Mandelstam 1960).

As Table 2 shows, uptake rates for sugar and amino acid in mouse intestine are about 7 times higher by the sleeve method than by the ring method, and are twice as high in rat intestine by the sleeve method as by the chamber method. Apparently, the most nearly similar uptake rates for the same or similar solutes are those of Ramaswamy et al. (1980), who obtained uptakes of two monosaccharides by mouse intestine 69% of our values, using a sleeve technique similar to ours but using a different strain of mouse. We think that two important reasons for the high uptake rates in sleeves may be improved stirring and reduced tissue damage. The stirring obtained with a stirring bar at 1,200 rpm a few mm from the sleeve is similar to the best stirring obtained in chambers, and much superior to stirring possible with intestinal rings or sacs. Stirring is important because the diffusion resistance of an unstirred layer can reduce uptake rates manyfold (cf. Figs. 4 and 5), and also because oxygenation may be critical. Tissue damage is minimal with sleeve preparations, because tissue handling is minimal except for everting the intestine.

Further advantages of the sleeve technique are that it permits use of very small vertebrates whose intestines are difficult to mount in chambers; it maintains the tissue in a fixed configuration, unlike rings; it readily permits extrapolation to the whole- animal level, by measuring transport per cm of intestine in sleeves taken from several different po- sitions along the gut; and it involves simpler equipment than chambers, so that one person can measure uptake in a dozen preparations within a few hours.

Unstirred layers

Consider a flat membrane separated from a well-stirred bathing solution by an unstirred layer of thickness δ, whose value depends on the stirring rate. If there is a net solute flux across the membrane because of active transport or else because of passive permeation down a concentration gradient, the solute concentration immediately at the membrane will differ from that in the bulk solution. Hence, if one calculates the passive permeability constant P or Michaelis-Menten constant K_m from the measured flux and the bulk concentration, the resulting apparent permeability coefficient P* or Michaelis-Menten constant K_m* will differ from the true values P or K_m. The relations are (from Winne 1973)

\[
1/P = (1/P*) - R, \tag{1}
\]

\[
K_m = K_m* - 0.5 J_{max} R \tag{2}
\]

where R, the diffusional resistance of the unstirred layer, is given by

\[
R = \delta/D, \tag{3}
\]

and where D is the solute's diffusion coefficient. For a flat membrane, fluxes J are easily expressed with respect to membrane area, so that J and J_{max} have units of moles/cm²·s and P has units of J divided by concentration (i.e., cm/s).

In elaborately folded membranes such as the intestine, the problem is far more complex (Winne
1973; Lukie et al. 1974; Westergaard and Dietschy 1974; Thomson and Dietschy 1977, 1980a, b). The effective area of membrane greatly exceeds the nominal area because of villi and microvilli and is not easy to determine. It is still harder to determine whether solute transport is uniformly distributed over villous and microvillous folds. Hence intestinal fluxes are usually expressed with respect to some measure other than area, such as tissue dry weight. Equation 3 then becomes

\[ R = \delta/DA \]  

(4)

where \( A \) is the effective membrane area for uptake, per mg dry weight or per cm\(^2\) nominal area depending on the units in which fluxes are expressed. Not only \( \delta \) but also \( A \) vary with stirring rate (cf. Table IV of Westergaard and Dietschy 1974). Worse yet, \( \delta \) and \( A \) vary between solutes depending on their sites of uptake along the villus.

A method used to estimate the unstirred-layer resistance \( R \) for intestine is from uptake rates of highly permeant probes for which the unstirred layer may be assumed rate-limiting \( [1/P^* \sim R \gg 1/P] \) in Eq. (1). By this method we can compare \( R \) for our sleeve preparation of mouse jejunum with \( R \) for a well-stirred chamber preparation of rabbit jejunum (Westergaard and Dietschy 1974). The latter authors, using the highly permeant probe decanol, calculated that \( R \) in rabbit jejunum decreases 13-fold as the stirring rate increases from 0 to 800 rpm. At 800 rpm they obtained \( \delta = 0.0137 \text{ cm}, \text{effective area (} S_m \text{ in their terminology) } 3.7 \text{ cm}^2 \text{ per cm}^2 \text{ nominal surface area, which combined with } D_{\text{decanol}} = 9.7 \times 10^{-6} \text{ cm}^2/\text{s} \text{ at } 37 \text{ °C yields } R_{\text{decanol}} = (0.0137)/(3.7)(9.7 \times 10^{-6}) = 381 \text{ s cm}^3/\text{cm}^2 \text{ nominal area/cm}^3 \text{. In mouse jejunal sleeves at 1,200 rpm the measured decanol permeability may be estimated from the uptake at 30 s (Fig. 5), assuming uptake then still to be linear with time (hence probably underestimating } P^* \text{ and overestimating } R, \text{ as } 296 \text{ μl/min·cm length. Assuming } 1.57 \text{ cm}^2 \text{ nominal area per cm length for a rod diameter of } 4 \text{ mm and sleeve outer diameter of } 5 \text{ mm, assuming decanol uptake to be unstirred-layer limited (} . . P^* \sim DA/\delta \), and taking } D_{\text{decanol}} \text{ as } 9.2 \times 10^{-6} \text{ cm}^2/\text{s} \text{ at } 37 \text{ °C yields } R_{\text{decanol}} \sim 1/\text{P}^* \text{ = 320 s cm}^{-2} \text{ nominal area/cm}^2 \text{. Even this overestimate for the sleeves is lower than the chamber value and indicates that control of unstirred layers is at least as good in sleeves as in chambers. However, we are still not permitted to insert our } R_{\text{decanol}} \text{ estimate into Eq. (2) in order to calculate the true } K_m \text{ for glucose, because we do not know whether glucose and decanol are taken up at the same site along the villus and hence whether they have the same } R \text{ value. It remains an open question whether unstirred layers cause measured } K_m^* \text{'s in even the best-stirred chamber and sleeve preparations to be serious overestimates of the true } K_m \text{ values.}

**Application of the sleeve technique to other vertebrate species**

We have found the sleeve technique applicable to a wide variety of vertebrate species, ranging in size from 5-gram hummingbirds to 1-kg owls. To illustrate the results, Table 3 gives measurements of D-glucose and L-proline uptake in intestines of four species representing four vertebrate classes. As an example of the conclusions that such measurements can yield, note that uptake rates per unit tissue in the iguana are comparable to those in a similar-sized mammal, the mouse, despite the 10-fold higher metabolic rates and fuel requirements of mammals than lizards. Uptake measurements in other lizard and mammal species extend this conclusion. Hence high uptake rates per unit of intestine are not the explanation for the much greater nutrient extraction rates of mammals. Instead, the intestine is longer and has a much higher food processing rate in mammals than in similar-sized lizards. In other papers (Karsov and Diamond, in preparation) we use the sleeve technique to examine phenotypic, developmental, and evolutionary responses of intestinal transport to changes in diet and metabolic demand in vertebrates.

**Final caveats**

The method described in this paper is appealing in its technical simplicity and in the range of species to which it can potentially be applied. Knowledge of intestinal nutrient uptake is important in understanding numerous physiological and ecological problems. Lest readers be misled into applying the method hastily, we emphasize the controls that should be performed, if measurements of intestinal uptake by this method or any other method are to be meaningful:

1. Use PEG as in Fig. 2a, to determine the rate at which the unstirred layer equilibrates.
2. Determine a suitable rinse time as in Fig. 1.
3. Measure PEG transfer to the serosal fluid as a check for edge damage, and measure transfer of the test solute to the serosal fluid in order to set an upper limit on the choice of incubation time.
4. Determine the length of the linear uptake phase for the test solute, as in Fig. 2B and C. The
Table 3. Uptake of 0.5 mM D-glucose and 0.5 mM L-proline by everted intestinal sleeves of four vertebrate species. Measurements were made at 37 °C for the mammal, bird, and reptile, at 23 °C for the fish. Uptake is expressed as nmoles/min·unit tissue, where tissue units are taken as either mg dry weight, cm length of intestine, or cm² nominal surface area (i.e., neglecting multiplication of area by villous folds and microvilli). D-glucose uptake is active uptake only (i.e., total uptake corrected for adherent fluid and passive uptake by subtracting tissue D-glucose), while L-proline uptake is active plus passive uptake (i.e., total uptake corrected for adherent fluid by subtracting tissue PEG). Errors are standard errors of the mean. The third column is the diameter of glass rod used to give a good fit to the intestinal diameter. 14C D-glucose was used for each species except sturgeon, where tissues were incubated in 3H D-glucose but not dried prior to counting. Incubation times were as follows. Mouse and shrike: D-glucose 1 min, L-proline 2 min. Iguana: both solutes 4 min. Sturgeon: both solutes 8 min (uptake was linear with time for 16 min).

<table>
<thead>
<tr>
<th>Species</th>
<th>Body weight (g)</th>
<th>Rod diameter (mm)</th>
<th>D-glucose, active uptake</th>
<th>L-proline, total uptake</th>
<th>Region of intestine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Laboratory mouse (Mus musculus)</td>
<td>38</td>
<td>4</td>
<td>9.3 ± 0.8 (5)</td>
<td>3.5 ± 0.8 (5)</td>
<td>Jejunum</td>
</tr>
<tr>
<td>Loggerhead shrike (Lanius ludovicianus)</td>
<td>48</td>
<td>3</td>
<td>0.20 ± 0.6 (3)</td>
<td>0.30 ± 0.10 (3)</td>
<td>Proximal gut</td>
</tr>
<tr>
<td>Desert iguana (Dipsosaurus dorsalis)</td>
<td>75</td>
<td>6</td>
<td>7.9 ± 1.0 (3)</td>
<td>7.2 ± 1.2 (7)</td>
<td>Proximal-mid gut</td>
</tr>
<tr>
<td>Sturgeon (Acipecten transmontanus)</td>
<td>75</td>
<td>4</td>
<td>0.08 ± 0.02 (2)</td>
<td>0.07 ± 0.01 (6)</td>
<td>Mid gut</td>
</tr>
</tbody>
</table>

length of this phase may decrease at high solute concentration and may differ among solutes.

5. Check whether errors arising from solute metabolism to a volatile product have been eliminated.

For any extended study of uptake in a particular species, all five of these control experiments should be performed in that species. If one simply desires a few uptake values in a particular species, one may perhaps be able to omit controls 1, 2, 3, and 5 and instead to use the procedures that we validated for mice. However, it remains essential to carry out the fourth control experiment anew with each species.

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