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A Method for Measuring Apical Glucose Transporter Site Density in Intact Intestinal Mucosa by Means of Phlorizin Binding

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Summary. Phlorizin binding has been widely used to estimate the site density of glucose transporters on intestinal and renal brush-border vesicles. Glucose transport measurements in the intact intestinal mucosa show that changes in transport rate postulated to arise from changes in site density occur under many physiological and pathological conditions. Exploring the basis of these regulatory phenomena would be facilitated by comparing changes in transport rate and site density measured in the same preparation. Hence we developed methods for measuring phlorizin binding in everted sleeves of intact mouse intestine. Specific binding of phlorizin to glucose carriers reached an asymptotic value within 120 sec, while nonspecific binding continued to rise thereafter. Hence we used 120-sec incubations. The rate of dissociation of specifically bound phlorizin was accelerated by Na^+-free solutions and even more by 50 mM glucose, while the rate of dissociation of nonspecifically bound phlorizin was independent of these solution changes. Hence we chose a 20-sec rinse in Ringer + 50 mM mannitol, because it washes out 30–40% of the nonspecifically bound phlorizin but virtually none of the specifically bound phlorizin. Ligand-binding analysis of specific binding against phlorizin concentration suggested two classes of binding sites, of which the one with stronger affinity for phlorizin probably has the higher capacity for glucose transport in mouse jejunum. The calculated affinity and capacity of this component are independent of whether one estimates the specific component of total binding by adding glucose or by removing Na^+.

Key Words small intestine · phlorizin binding · dietary regulation · glucose transport · induction

Introduction

The plant glycoside phlorizin is widely used as a selective, membrane-impermeant, competitive inhibitor of active D-glucose transport in the brush border (apical membrane) of small intestine and kidney, because phlorizin has a much greater affinity for the glucose carrier than does glucose itself (Alvarado & Crane, 1964; Diedrich, 1966; Stirling & Kinter, 1967; Ullrich, 1979). Phlorizin binding thus serves as a measure of glucose transport site density. Phlorizin itself is not transported (Silverman, 1974; Silverman & Black, 1975). Most studies of phlorizin binding have been done on brush-border membrane vesicles.

Regulatory biologists have observed that intestinal glucose transport rates respond reversibly to many physiological conditions such as pregnancy, lactation, and high dietary carbohydrate levels, and to signals such as hormones (*see* Karasov & Diamond, 1983*a*, 1986, for reviews). It has been inferred that some of these responses depend on specific changes in glucose transport site density, but proof is lacking. The most direct way to assess this hypothesis quantitatively would be to employ phlorizin for measuring binding site density in the same preparation of intact intestinal mucosa used for measuring regulatory changes in transport rate.

Hence the purpose of this paper is to adapt methods of measuring phlorizin binding in vesicles for use on the intact intestinal mucosa. The main practical problems we encountered were (1) to identify an incubation time, rinse time, and rinse conditions optimal for distinguishing specific from nonspecific binding; and (2) to assure ourselves that alternative estimates of phlorizin binding site density based on blocking sites with glucose or on inhibiting binding with Na^+-free solutions were in agreement. The methods developed in this paper will then be used in the following paper (Ferraris & Diamond, 1986) to understand the basis of two familiar phenomena in intestinal regulation: the dietary dependence of glucose transport, and the gradient in glucose transport along the length of the intestine.

Materials and Methods

Animals and Diet

Adult male Swiss Webster mice (30–40 g) were housed in cages with constant access to water and chow (Wayne Lab-Blox) for at

least two weeks before sacrifice. Weight gained and amount of food consumed were measured twice a week for each mouse. Initial weight (average ± SEM) was 35.3 ± 0.7 g (n = 35); weight at sacrifice, 39.3 ± 1.0 g (35); daily food consumption, 4.5 ± 0.3 g (35). All mice gained weight and appeared healthy.

Solutions

The composition of normal Ringer's solution (in mM) was: 128 NaCl, 4.7 KCl, 2.5 $CaCl_2$, 1.2 $MgSO_4$, 4 KH_2PO_4, 16 $NaHCO_3$; osmolarity 290 mOsm; pH 7.0, gassed with 95% O_2–5% CO_2. Na^+-free solutions were obtained by equimolar replacement of NaCl and $NaHCO_3$ with choline chloride and choline bicarbonate. Solutions containing 50 mM glucose or mannitol were prepared by isosmotic replacement of NaCl.

Phlorizin Uptake Measurements

Uptake measurements were based on the methods of Karasov and Diamond (1983*b*). Briefly, a mouse was anesthetized by intraperitoneal injection of 0.1 ml nembutal, and the small intestine was excised, transferred to ice-cold Ringer, and everted. A 1-cm everted sleeve of upper jejunum (the region where glucose transport is maximal) was then mounted on a solid glass rod, preincubated in Ringer at 37°C for 5 min, and incubated at 37°C in flat-bottomed test tubes with 12 ml incubation medium containing ^{3}H-phlorizin and ^{14}C-polyethylene glycol (PEG, mol wt 4000). To minimize effects of unstirred layers, solutions were stirred at 1200 rpm by a magnetic bar and bubbled with 95% O_2, 5% CO_2. After a brief rinse whose duration and composition will be discussed, the tissue was weighed in a tared vial, dissolved in tissue solubilizer (TS-1, Research Products International), and counted. ^{14}C-PEG was used as an extracellular space marker to correct total ^{3}H counts for counts in adherent fluid and thereby to obtain the tissue-associated ^{3}H-phlorizin. Solution pH was regularly monitored before and after each incubation, because phlorizin binding is inhibited above pH 7.5 (Toggenburger, Kessler & Semenza, 1982).

To minimize any decrease in phlorizin concentration of the incubation medium due to binding by the tissue, incubation solutions were used only once and were large (12 ml) relative to the tissue (17–60 mg). Furthermore, the initial and final ^{3}H activity of the incubation medium was regularly measured, and in some cases a sample of medium was analyzed by thin-layer chromatography (*see below*). The decrease in ^{3}H activity during the incubation was by only 2.55 ± 0.37% (n = 32) for glucose-containing and Na^+-free media, by 2.08 ± 0.67 (n = 32) for mannitol-containing media and normal Ringer, and each of these values was independent of phlorizin concentration ($P > 0.40$) from at least 0.005 to 1.28 μM.

Phlorizin Washout Measurements

To select optimal choices of rinse medium and rinse time, tissues were preincubated at 37°C for 5 min, incubated in 0.04 μM ^{3}H-phlorizin at 37°C for 2 min, and rinsed in 12 successive 25-ml aliquots of ice-cold Ringer stirred at 1000 rpm for periods progressively increasing from 5 to 360 sec, for an accumulated rinse time of 900 sec. A 2-ml aliquot of each rinse solution was counted in 10 ml of liquid scintillation cocktail for aqueous samples (PCS, Amersham), and the cumulative DPM rinsed from the tissue was calculated. After the last rinse the tissue was dissolved and counted as usual.

Thin-Layer Chromatography

To determine whether phlorizin hydrolysis into phloretin and glucose occurred during incubations, everted intestinal sleeves from mice fed high- and low-carbohydrate diets (*see* Ferraris & Diamond, 1986, for composition) were incubated in 0.005 or 1.28 μM phlorizin, rinsed, and extracted in 2 ml absolute ethanol for at least 24 hr. Ethanol extracts of the tissue and also of the incubating solution were then analyzed by thin-layer chromatography with two systems: a nonpolar silica gel system whose solvent consisted of ethanol/chloroform/butanol (26:60:4 vol/vol), and a polar cellulose system whose solvent consisted of ethanol/acetic acid/water (20:1:80 vol/vol; Diedrich, 1968). In the former, phloretin travelled close to the solvent front (R_f = 0.9) while phlorizin stayed close to the origin (R_f = 0.1); the second system effected the opposite migration pattern (R_f for phloretin = 0.1, phlorizin = 0.6). ^{3}H-phlorizin was used as a standard and was spotted along with unlabeled phlorizin and phloretin. Phlorizin and phloretin were detected with 2.7% $FeCl_3$ in 2 M HCl and 10% phosphomolybdic acid in ethanol (Sigma).

Chromatograms were quantitatively analyzed by cutting up 0.75 × 1 cm squares. These plastic squares were placed in plastic scintillation minivials containing 0.5 ml of absolute ethanol to solubilize absorbed phenols, vortexed, extracted for 3 hr, then counted in 5 ml Econoflour. Samples of extracts were also counted in separate scintillation vials to estimate recovery rates. Mean recovery rate for samples from both the nonpolar and the polar systems was 78 ± 6% (n = 16).

Statistical Analysis

Results are expressed as mean ± SEM (n = sample size) or as regression estimate ± SE of the estimate. Nonlinear regression estimates were obtained by unweighted least squares using program BMDPAR (Dixon, 1985). For ligand binding analysis, log transforms of the observed and predicted binding were used in the fitting to ensure constant variance of the residual errors. The Akaike and Schwarz criteria or sequential F tests (Akaike, 1974; Schwarz, 1978; Landaw & DiStefano, 1984) were used to pick the most appropriate order of a sum-of-exponentials model for washout curves or the most appropriate number of binding sites for the ligand-binding data. Analysis of variance or the t test was used to judge the significance of differences among or between experimental treatments.

Chemicals

^{3}H-phlorizin, ^{3}H-L-glucose, ^{14}C-D-glucose, and ^{14}C-polyethylene glycol were purchased from New England Nuclear, while other chemicals were from Sigma. The ^{3}H-label in the phlorizin molecule is in the phenyl rings of the aglycon moiety, allowing detection of phloretin during thin-layer chromatography.

Results

Choice of Incubation Time

The optimal choice of incubation time depends on several trade-offs. First, although equilibrium binding of phlorizin is rapid in whole tissue (Diedrich, 1968), the incubation should still be long enough to

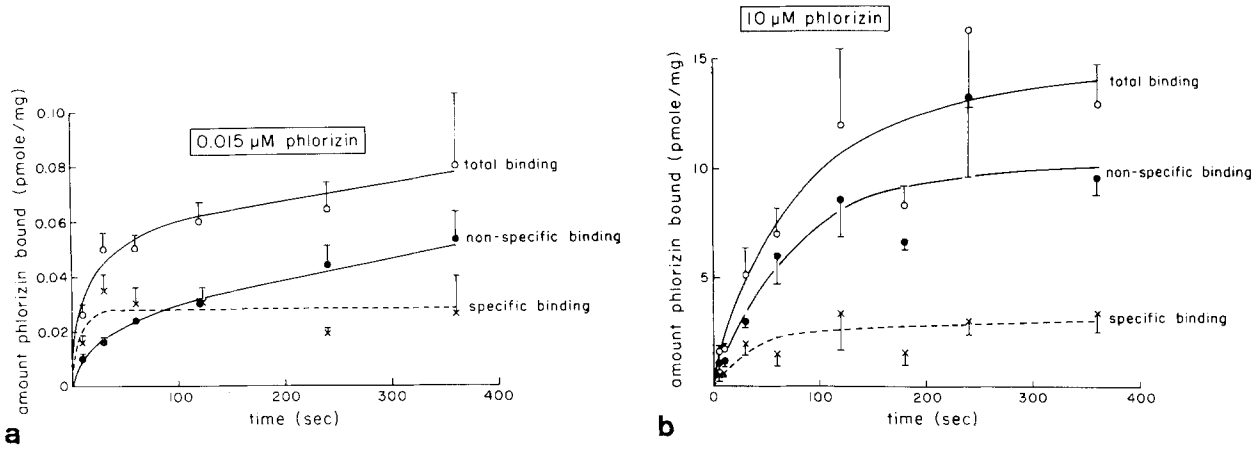


Fig. 1. Tissue uptake of ^{3}H-phlorizin as a function of incubation time. ○, total binding (i.e., binding in Ringer + 50 mM mannitol); ●, nonspecific binding (i.e., binding in Ringer + 50 mM D-glucose); ×, specific binding to glucose-protectable sites (calculated as points ○ minus points ●). Phlorizin concentration, 0.015 μM (*a*) or 10 μM (*b*). Values are mean ± SEM ($n = 5$). Specific binding curves are least-squares nonlinear fits of the data to an exponential plus an asymptote [Eq. (1)]; total and nonspecific binding curves include a linear component

allow complete equilibration of the adherent fluid with both phlorizin and PEG in the bathing solution, thereby permitting an accurate correction for phlorizin in the adherent fluid. PEG equilibrates within 2 min in everted sleeves of mouse intestine (Karasov & Diamond, 1983*b*), and phlorizin must equilibrate more rapidly because of its lower molecular weight. Second, the incubation should be brief enough that phlorizin hydrolysis to phloretin is minimal, thereby reducing errors caused by phloretin binding and uptake. Finally, and most important, phlorizin is bound not only specifically to the glucose transporter, but also nonspecifically to other tissue components. If these two components of binding have different time courses, we should seek an incubation time that maximizes the specific relative to the nonspecific component.

Hence we incubated sleeves with either 0.015 or 10 μM phlorizin for 5, 10, 30, 60, 180, 360, and 600 sec, doing parallel incubations in Ringer + 50 mM D-mannitol and in Ringer + 50 mM D-glucose. The incubation was concluded by rinsing the tissue for 20 sec in ice-cold normal Ringer (Karasov & Diamond, 1983b). Binding in D-mannitol should represent total binding (specific plus nonspecific). However, 50 mM D-glucose (a 5000-fold or 3,000,000-fold excess of glucose over phlorizin) should block the glucose transporter sites, leaving only the nonspecific component of binding. Binding in mannitol minus binding in D-glucose should represent the specific component of binding.

Figure 1 depicts the time courses of total, nonspecific, and specific binding. We assumed that specific binding reaches a level asymptote exponentially and proceeded to analyze the data according to the exponential model:

$$S_t = p_1(1 - e^{-t/p_2}) \quad (1)$$

where S_t is the specific binding (= total − nonspecific binding) after t seconds incubation, and parameters p_1 and p_2 are the asymptotic binding and time constant, respectively. The time to reach a fraction α of the asymptote ($0 < \alpha < 1$) is computed by

$$t_\alpha^* = p_2[-\text{in}\,(1 - \alpha)]. \quad (2)$$

For Fig. 1*a* and *b*, the regression estimates of the p_1's were 0.028 ± 0.003 and 3.00 ± 0.31 pmoles/mg, respectively, while p_2's were 9.0 ± 5.6 and 46 ± 18 sec, respectively. Thus, the time to reach 90% of the asymptote would be 21 ± 13 sec for the 0.015 μM phlorizin concentration, and 105 ± 42 sec for 10 μM.

Nonspecific binding (and hence also total binding) continues to increase after 120 sec. When we fit the data at 60–360 sec to straight lines, the slope for specific binding did not differ significantly from 0, either at 0.015 or 10 μM. Correspondingly, the slopes for total and nonspecific binding, while significantly different from zero, did not differ significantly from each other: 0.020 ± 0.010 (20) *vs.* 0.015 ± 0.008 (20) pmol/mg, sec at 10 μM, 0.076 ± 0.042 (21) *vs.* 0.101 ± 0.021 (21) fmol/mg, sec at 0.015 μM. This simply confirms one's visual impression that specific binding essentially reaches an asymptote by 120 sec, hence that the increase in total binding with time thereafter parallels the increase in nonspecific binding.

We therefore chose an incubation time of 120 sec as the best compromise to ensure completeness of specific binding to glucose transporters while minimizing nonspecific binding.

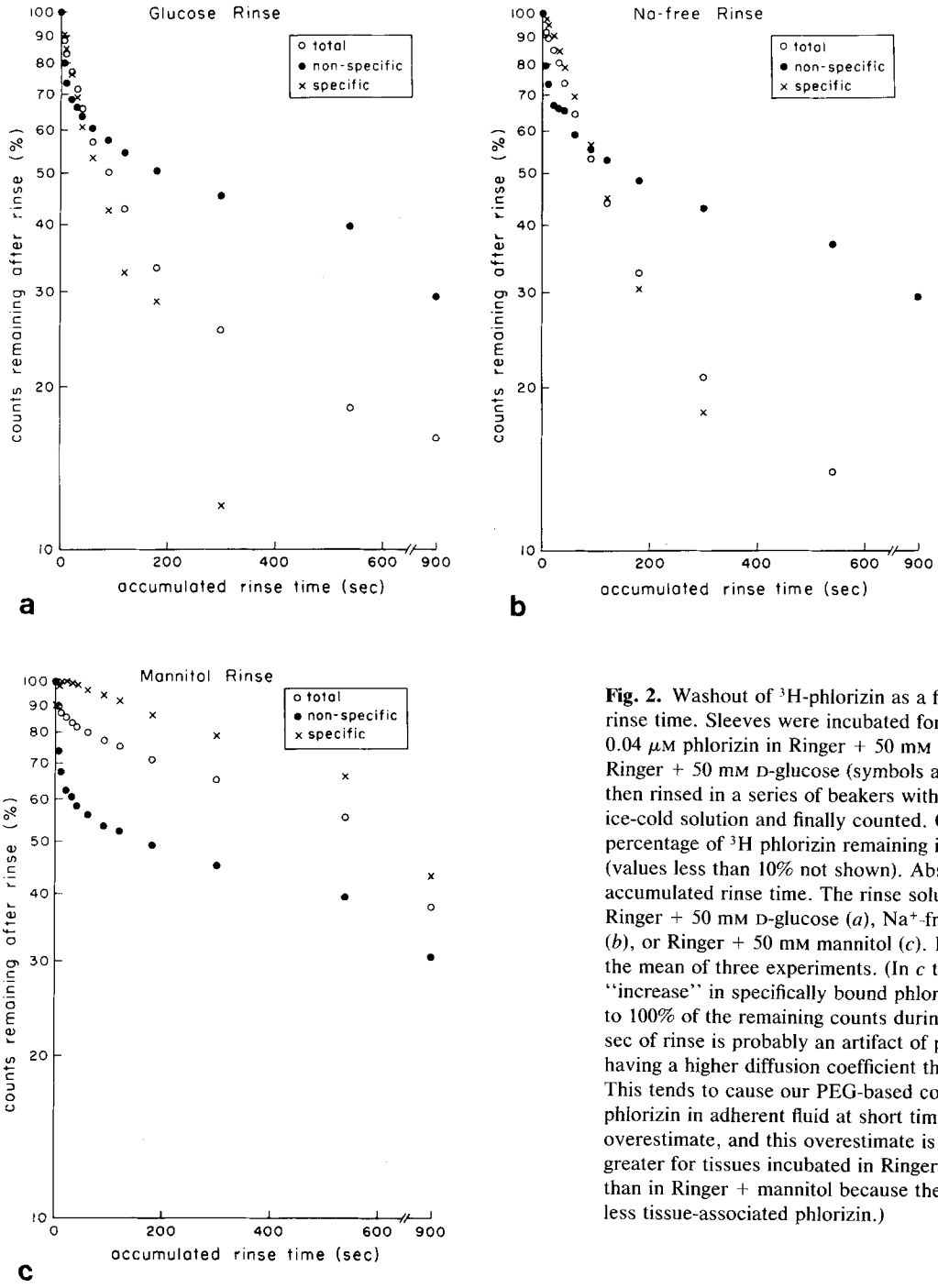


Fig. 2. Washout of ^{3}H-phlorizin as a function of rinse time. Sleeves were incubated for 2 min with 0.04 μM phlorizin in Ringer + 50 mM mannitol or Ringer + 50 mM D-glucose (symbols as in Fig. 1), then rinsed in a series of beakers with 25 ml of ice-cold solution and finally counted. Ordinate: percentage of ^{3}H phlorizin remaining in the tissue (values less than 10% not shown). Abscissa: accumulated rinse time. The rinse solutions were Ringer + 50 mM D-glucose (*a*), Na^+-free Ringer (*b*), or Ringer + 50 mM mannitol (*c*). Each point is the mean of three experiments. (In *c* the apparent "increase" in specifically bound phlorizin from 90 to 100% of the remaining counts during the first 20 sec of rinse is probably an artifact of phlorizin having a higher diffusion coefficient than PEG. This tends to cause our PEG-based correction for phlorizin in adherent fluid at short times to be an overestimate, and this overestimate is relatively greater for tissues incubated in Ringer + glucose than in Ringer + mannitol because the former have less tissue-associated phlorizin.)

Choice of Rinse Solution and Time

The purpose of a brief cold rinse after incubation is to reduce the amount of phlorizin that is present in the adherent fluid and that must be corrected for. The longer the rinse, the more of this adherent phlorizin will be removed, but the rinse should also not be so long that it removes phlorizin bound to the membrane. We already know that a 20-sec rinse removes 72% of the counts due to PEG (all of which is in the adherent fluid) but only 2% of the counts due to tissue-associated D-glucose (Karasov & Diamond, 1983*b*). Ideally, we would also like to choose a rinse medium that removes as much as possible of the nonspecifically bound phlorizin, and as little as possible of the specifically bound phlorizin. In addi-

Table 1. Effect of rinse medium on efflux of nonspecifically and specifically bound phlorizin in mouse jejunum

	Nonspecific binding			Specific binding		
Parameter	Glucose	Na^+-free	Mannitol	Glucose	Na^+-free	Mannitol
A_1 (%)	25.7 ± 1.2[a]	26.7 ± 1.1[a]	30.6 ± 1.0[a]	4.54 ± 2.48	100.0	100.0
λ_1 (sec^{-1})	−0.247 ± 0.025[b]	−0.235 ± 0.022[b]	−0.313 ± 0.020[b]	−0.603 ± 1.8	−0.0063 ± 0.0002[i]	−0.00089 ± 0.00003[j]
A_2 (%)	17.4 ± 1.1[c]	21.8 ± 1.4[c]	13.7 ± 0.8[c]	33.2 ± 9.6	—	—
λ_2 (sec^{-1})	−0.017 ± 0.003[de]	−0.012 ± 0.002[d]	−0.028 ± 0.003[e]	−0.0250 ± 0.0085	—	—
A_3 (%)	56.9 ± 1.1[f]	51.5 ± 1.6[f]	55.6 ± 0.4[f]	62.3 ± 10.8	—	—
λ_3 (sec^{-1})	−0.000717 ±0.000036[g]	−0.000607 ±0.000051[g]	−0.000666 ±0.000015[g]	−0.00553 ± 0.00081[i]	—	—
Lag time (sec)	—	—	—	—	—	19.8 ± 7.4
MRTRD (sec)	804 ± 28[h]	868 ± 50[h]	842 ± 15[h]	126 ± 6[k]	160 ± 4[l]	1142 ± 36[m]
Median residence time (sec)	197	157	164	65	111	798

Numbers represent regression estimates ± SE of the estimate from fitting the data of Fig. 2. A_i's and λ_i's are proportions and exponential time constants as defined in Eq. (3), text. Sum of A_i's is constrained to equal 100%. Lag time = time elapsed before a significant amount of tracer was rinsed off from binding sites, as defined by Eq. (3). MRTRD = mean of residence time distribution; median of residence time distribution represents time elapsed after half of the tracer washes out. When comparing columns, values with the same superscript in the same row are not significantly different from each other ($P > 0.05$). For specific binding, λ_3 in Ringer + glucose was compared to λ_1's of Na-free Ringer and Ringer + mannitol rinses. In the 5th and 6th columns (glucose and Na-free rinses) only data from 0–360 sec were used in the calculations, because percent tracer left was already 0 for $t > 360$ sec in some tissues rinsed in these media.

tion, the cold rinse should stop tissue metabolism of phlorizin. Hence we compared three alternative rinse solutions: Ringer + 50 mM D-glucose, Na^+-free Ringer, and Ringer + 50 mM mannitol. Figure 2*a–c* depicts the resulting washout curves, while Table 1 gives calculated efflux constants.

Even without a quantitative analysis, inspection of Fig. 2*a–c* yields an obvious conclusion: washout of specifically bound phlorizin is greatly slowed in the mannitol rinse solution compared to the other two rinse solutions, while washout of nonspecifically bound phlorizin proceeds at similar rates in all three rinse solutions. In the mannitol rinse, specifically bound phlorizin washes out much more slowly than nonspecifically bound phlorizin. In the other two rinses this advantage is modest at short times (40–60 sec) and is reversed at longer times. Since the purpose behind the experiment of Fig. 2 was to choose a rinse solution that removes much of the nonspecifically bound phlorizin and little of the specifically bound phlorizin, Ringer + 50 mM mannitol is clearly the preferred rinse. For practical purposes, note that a 20-sec rinse removes 30–40% of the nonspecifically bound phlorizin in all three solutions, and removes 22 and 12% of the specifically bound phlorizin in Ringer + glucose and Na^+-free Ringer, respectively, but removes essentially no specifically bound phlorizin in Ringer + mannitol. Hence in all subsequent experiments we chose a 20-sec rinse in Ringer + 50 mM mannitol in order to reduce nonspecific binding without affecting specific binding.

Quantitative analysis of Fig. 2 suggests some further conclusions. We assumed that the washout curves in Fig. 2*a–c* reflect an exponential decay, or the sum of several exponentials, and proceeded to analyze the data by nonlinear regression methods according to the equation:

$$R_t = \sum_{i=1}^{n} A_i e^{-\lambda_i (t-L)}, \; t > L \tag{3}$$

where R_t is the percent tracer bound to tissue at time t, and the model parameters are A_i (percent tracer washed out under component i), λ_i (time constant [sec^{-1}] of component i), and L (lag time). The parameter estimates were constrained to be positive and to satisfy $\Sigma_{i=1}^{n} A_i = 100$. L was estimated only for the specific binding washout with the mannitol rinse; for all other washout data, L was fixed at zero. The "best" model order, n, was determined by the Akaike information criterion; this generally agreed with the order picked by the Schwarz criterion or sequential F tests. The median residence time of bound phlorizin was estimated by the time at which the predicted value of R_t is 50%. The mean residence time for "residue detection" (MRTRD: Landaw & Katz, 1985) was estimated by

$$\text{MRTRD} = \left\{ \left[\sum_{i=1}^{n} (A_i/\lambda_i) \right] \Big/ \left(\sum_{i=1}^{n} A_i \right) \right\} + L. \tag{4}$$

The washout of total binding was not analyzed, since it was merely the sum of the nonspecific and specific washout curves.

Figure 2 and Table 1 show that efflux of nonspecifically bound phlorizin in all three rinse solutions cannot be described by a single exponential but that three exponentials are required in every case. Solu-

Table 2. Effect of incubation procedure on properties of phlorizin binding sites in mouse jejunum

	Na^+ & Na^+-free	Mannitol & glucose	P value
High-affinity site			
K_{d1} (μM)	0.16 ± 0.03	0.17 ± 0.06	>0.50
R_1 (pmol/mg)	0.76 ± 0.11	0.89 ± 0.22	>0.50
R_1/K_{d1} (pmol/mg μM)	4.9 ± 0.3	5.2 ± 0.8	>0.50
Low-affinity site			
R_2/K_{d2} (pmol/mg μM)	0.67 ± 0.05	0.12 ± 0.05	<0.001

The table gives regression estimates and SE of the estimate for the receptor density (R) and dissociation constant (K_d) for site 1, and their ratio for site 2. The data fitted are those of Fig. 3. P values refer to the statistical significance of the difference between the values in the same row.

tion composition has no significant effect on the time constants of the fastest and slowest components, and on the A_i's of all components of nonspecific binding. For the second time constant there is a slight difference ($0.02 < P < 0.05$) between Na^+-free Ringer and Ringer + mannitol. MRTRD and median residence time of nonspecifically bound phlorizin are the same in all rinse media.

In the first 5 sec of rinse, the nonspecifically bound phlorizin is lost at an average rate of 3.6, 4.5, or 4.8%/sec, depending on the rinse medium. Most of this loss is coming from the fastest component. These rates are of the same order as that reported for the average washout rate for PEG (3.6%/sec, Karasov & Diamond, 1983*b*; the constant is somewhat lower for PEG than for phlorizin, as one would expect from their difference in molecular weights and hence in diffusion coefficients). This suggests that the fast component of nonspecific binding is limited only by diffusion in the unstirred layer and that solution composition has no influence on this diffusion. The slower components presumably reflect the slower dissociation of nonspecifically bound phlorizin from membrane lipids and proteins other than the glucose transporter; this process too is evidently independent of solution composition. Differences in unstirred-layer thickness between the tip and base of each intestinal villus could also contribute to the multi-exponential washout of nonspecifically bound phlorizin.

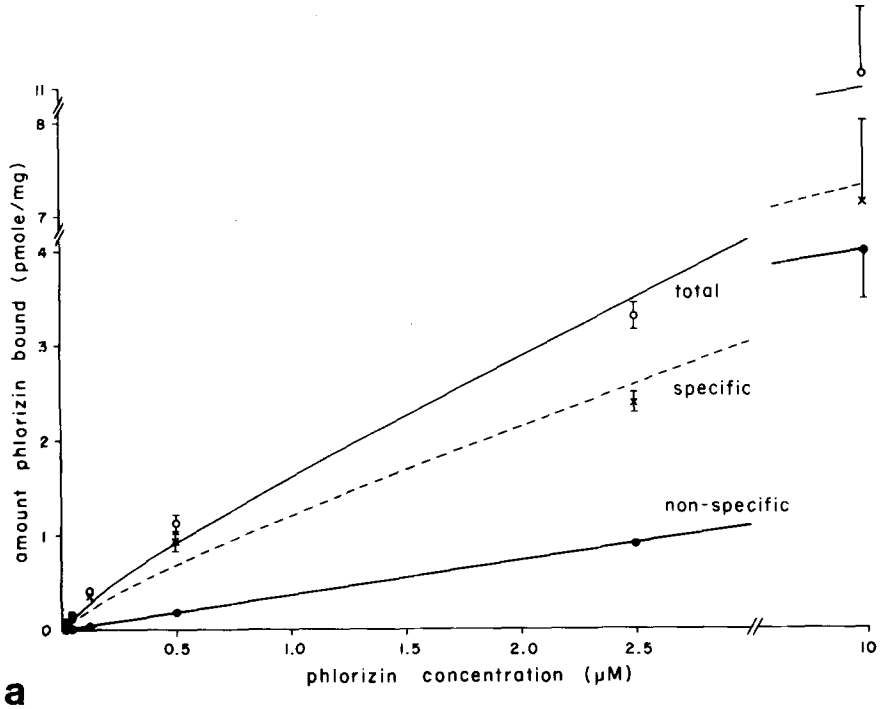
Turning our attention now to washout of specifically bound phlorizin, we note that median residence time and MRTRD are seven times briefer in Na^+-free Ringer than in Ringer + mannitol and are even briefer in Ringer + glucose (Table 2). Thus, either removal of Na^+ or else addition of glucose accelerates the dissociation of the phlorizin/transporter complex, just as one would expect for the Na^+-dependent binding of a competitive inhibitor of glucose transport. Washout is described by a single exponential in Ringer + mannitol or Na^+-free Ringer, by three exponentials in Ringer + glucose. As for the significance of the fact that description of the Ringer + glucose washout requires more exponentials, perhaps dissociation of phlorizin from the glucose transporter is rate-limiting in the other two rinse solutions, but is accelerated in Ringer + glucose to the point where the extracellular washout phase becomes detectable.

Choice of Incubation Solution

As already mentioned, a procedure for measuring specific phlorizin binding in vesicles has been to measure total binding in Ringer + mannitol, measure nonspecific binding in Ringer + D-glucose, and calculate specific binding as the difference. Another procedure has also been used: to measure total binding in normal Ringer, measure nonspecific binding in Na^+-free Ringer, and calculate specific binding as the difference (Toggenburger et al., 1978, 1982; Turner & Silverman, 1981). Hence we compared these two procedures in intact mucosal sleeves, to determine (hopefully) whether they would yield the same estimate of glucose transport site density, and whether one procedure had advantages that recommended it.[1]

Figure 3*a* depicts the concentration dependence of total, specific, and nonspecific binding by the Na^+/Na^+-free procedure, Fig. 3*b* by the mannitol/glucose procedure. Total binding increases rapidly at low concentrations and levels off to become a linear function of concentration at higher concentrations, with no significant difference between total binding measured by the two methods at concentra-

[1] A third procedure used in vesicles is to block access of labeled phlorizin to glucose carriers by high concentrations of unlabeled phlorizin (≥ 2.5 mM). We did not use this procedure because of poor solubility of phlorizin in Ringer at concentrations above 1 mM, complications introduced by the modest available specific activity of ^{3}H-phlorizin (supplied as a stock in absolute ethanol), and inhibition of ^{3}H-phlorizin binding by ethanol concentrations over 0.5% in the incubation medium.



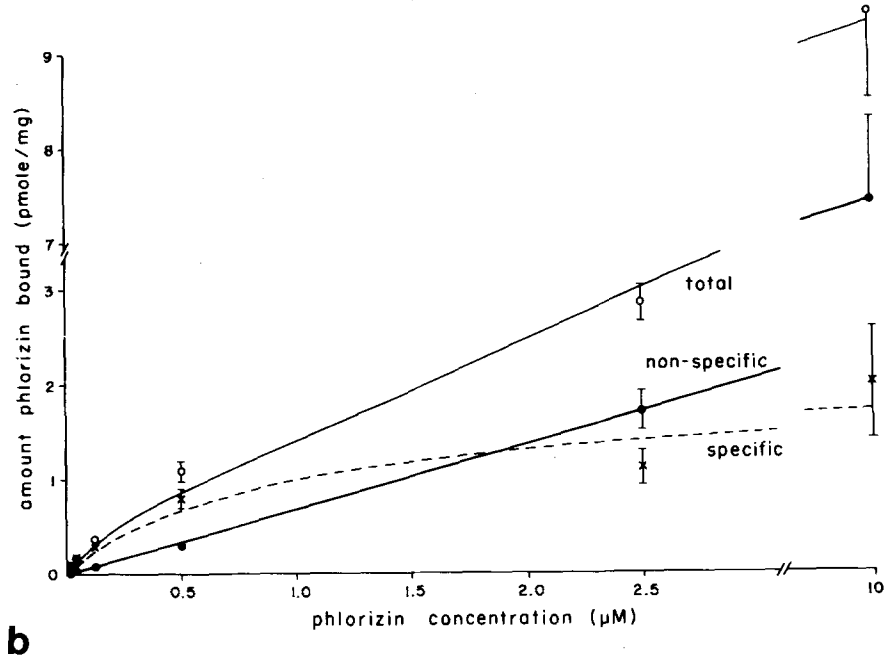


Fig. 3. Tissue uptake of ^{3}H-phlorizin as a function of concentration. ○, total binding, and ●, nonspecific binding, measured either as binding in Ringer and Na^+-free Ringer, respectively (*a*) or as binding in Ringer + 50 mM mannitol and Ringer + 50 mM D-glucose, respectively (*b*). ×, specific binding, calculated as points ○ minus points ●. Vertical bars show mean ± SEM. Each point is based on 4–6 determinations. Error bars are omitted if they are smaller than the plotted points

tions other than 2.5 μM. Nonspecific binding increases linearly with concentration, and the Na^+/Na^+-free and mannitol/glucose procedures yield equally good correlation coefficients (0.96 *vs.* 0.94) but somewhat different slopes ($P < 0.001$: 0.40 ± 0.02 (28) *vs.* 0.74 ± 0.05 (29) pmol/mg wet wt, μM phlorizin). Hence in the Na^+/Na^+-free procedure, where nonspecific binding is lower, specific binding exhibits a concentration dependence qualitatively similar to that of total binding (Fig. 3*a*): a hyperbolic increase at low concentrations, then a linear increase at high concentrations (Fig. 3*a*). In the glucose/mannitol procedure specific binding of phlorizin onto glucose-protectable sites is nearly saturated by 1 μM and rises more gently at high concentrations (Fig. 3*b*). Thus, the two procedures

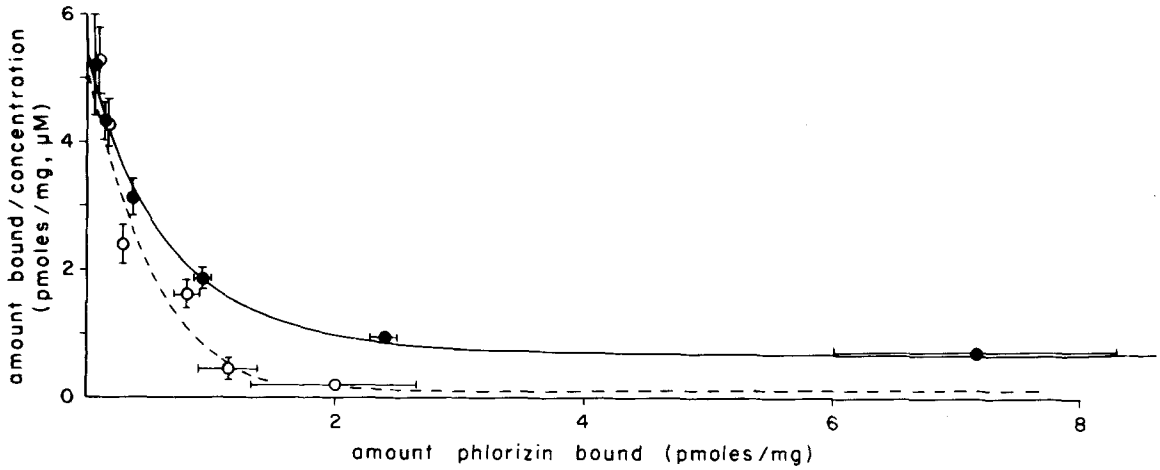


Fig. 4. Scatchard plot of equilibrium specific binding of phlorizin. Abscissa: amount bound. Ordinate: amount bound/phlorizin concentration. Points ● are based on points × of Fig. 3*a*; points ○, on points × of Fig. 3*b*. Each set of points would define a straight line if there were only one type of phlorizin binding site, but this is not true

yield the same estimates ($P > 0.1$) of specific binding at any given phlorizin concentration below 0.5 μM but yield differing estimates at 2.5 or 10 μM.

The data in Fig. 3*a* and *b* for specifically-bound phlorizin were analyzed by ligand-binding analysis, using nonlinear least squares (Rodbard, Munson & Thakur, 1980). We compared fits among one-, two-, and three-site models. For the data from both experimental procedures, the Akaike and Schwarz criteria selected as best a two-site model in which the lower affinity site is nonsaturable in the experimental range. This model is expressed by the following three-parameter equation:

$$\text{BOUND}/[\text{FREE}] = \{R_1/(K_{d1} + [\text{FREE}])\} + (R_2/K_{d2}) \quad (5)$$

where BOUND = amount of specifically-bound phlorizin (pmol/mg tissue); [FREE] = free phlorizin concentration (μM); R_1 or R_2 = receptor density (pmol/mg tissue) for high-affinity or low-affinity site, respectively; K_{d1} or K_{d2} = dissociation constant (μM) for high-affinity or low-affinity site, respectively. Although we cannot estimate R_2 and K_{d2} individually, we can distinguish their ratio from zero (Table 2). Using added phlorizin concentration (free + bound concentrations) as the independent variable, Eq. (5) was solved iteratively for BOUND as a function of the independent variable (Rodbard et al., 1980) for the least-squares procedure.

Transformation of the specific binding results to a Scatchard plot (Fig. 4) yields a nonlinear relation for the data from both experimental procedures, supporting the idea that specific binding involves at least two sites: site 1, which dominates at low phlorizin concentrations and has a ratio R_1/K_{d1} around 5; and site 2, which dominates at high phlorizin concentrations and has a ratio R_2/K_{d2} below 1.0 (Table 2). The two experimental procedures agree well in their estimates of site density R or dissociation constant K_d for site 1 but differ nearly sixfold in their estimates of R_2/K_{d2} for site 2. Since R_2/K_{d2} is much lower than R_1/K_{d1} by either procedure, site 2 must have a much lower site density or much higher dissociation constant (lower affinity) or both, compared to site 1. Whether or not site 2 proves to have a lower density than site 1, by definition it does have a lower affinity. K_d for our site 1 corresponds well to that of the high-affinity phlorizin binding site from kidney (Turner & Moran, 1982*a*,*b*).

In the following paper on effects of diet and intestinal position we determine the density and dissociation constant of only site 1: large statistical errors are inherent in our estimates for site 2, and the amount of intestinal tissue available from one animal restricted us to measuring phlorizin binding at only a few concentrations and hence to extracting constants for only one site. The following paper uses the mannitol/glucose procedure instead of the Na^+/Na^+-free procedure because of a much smaller confounding effect of the second site on constants determined for the first site. (Estimated specific phlorizin binding at the second site is only 4–11% of all specific phlorizin binding by the former procedure, but is six times greater by the latter procedure.)

Thin-Layer Chromatography

We measured hydrolysis of ^{3}H-phlorizin to ^{3}H-phloretin by intestinal sleeves at phlorizin concen-

trations of 0.005 or 1.28 μM, incubated either in Ringer + mannitol or in Ringer + glucose. The measurements were made on mice eating either a high-carbohydrate diet or a no-carbohydrate diet (*see* Ferraris & Diamond, 1986, for composition), yielding, respectively, high or low values of glucose transport rates. Since phloretin's percentage contribution to tissue ^{3}H counts proved to be independent of phlorizin concentration, results from the two concentrations were pooled. In no-carbohydrate mice, phloretin's contribution was 10.9 ± 2.6% (11) for Ringer + mannitol incubations, 11.9 ± 1.6% (11) for Ringer + glucose incubations. As these two values do not differ significantly ($P > 0.25$), Ringer + glucose incubations were omitted for high-carbohydrate mice; Ringer + mannitol incubations yielded 10.0 ± 2.3% (10) phloretin. Thus, only 10–12% of tissue radioactivity is in the form of phloretin.

We also measured phlorizin hydrolysis in the solutions in which tissues had been incubated. For high-carbohydrate mice, phloretin contributed 4.9 ± 1.0% (4) and 4.8 ± 0.8% (4) to solution radioactivity after Ringer + glucose and Ringer + mannitol incubations, respectively. Corresponding numbers for no-carbohydrate mice were 6.6 ± 2.5% (4) and 6.6 ± 3.3% (3). These four values do not differ significantly ($P > 0.40$).

Discussion

Time Dependence of Binding

In vesicle studies specific binding of phlorizin to the glucose transporter rapidly achieves a maximal value at 2–45 sec (Chesney, Sacktor & Kleinzeller, 1974; Peerce & Wright, 1984), thereafter declining slowly due to dissipation of the initial Na^+ gradient (Toggenburger et al., 1982) or to reduced binding (Glossmann & Neville, 1972). In the intact intestinal mucosa specific binding is slower, as one would expect from the much greater unstirred layers, but is nevertheless faster than nonspecific binding (Fig. 1). Specific binding reaches asymptotic values by 1 or 2 min, while equilibration of PEG is complete by 2 min (Karasov & Diamond, 1983*b*). Hence the time course of specific binding is probably limited in the intact mucosa by unstirred layers rather than by binding kinetics. We chose 2 min as the optimal incubation time because it is the shortest time that ensures equilibration of all relevant solutes (phlorizin, glucose, mannitol, PEG) with the adherent fluid. In addition, it is possible that specific binding decreases slowly in the intact mucosa after 2 min (Fig. 1*a*) as in vesicles, though our results do not make this clear.

Nonspecific Binding

With both the Na^+/Na^+-free and glucose/mannitol procedures, nonspecific binding proves to be directly proportional to phlorizin concentration. Because nonspecific binding accounts for more than 50% of total binding at higher concentrations but phloretin accounts for only 10–12% of total binding, phloretin is unlikely to contribute significantly to nonspecific binding. Presumably the nonspecific binding represents phlorizin bound to tissue components other than the glucose transporter. The amount of nonspecific binding is lower in Na^+-free Ringer (Fig. 3*a*) than in Ringer + glucose (Fig. 3*b*), but the rate of dissociation of nonspecifically bound phlorizin is independent of rinse solution composition (Table 1).

Specific Binding

Dissociation of specifically bound phlorizin from glucose transporters is accelerated by removal of Na^+ and accelerated even more by addition of D-glucose (Table 1). These results parallel results from vesicle studies, where D-glucose in the presence of Na^+ competitively inhibits phlorizin binding (Glossmann & Neville, 1972; Chesney et al., 1974; Silverman & Black, 1975; Tannenbaum et al., 1977; Toggenburger et al., 1978; Turner & Silverman, 1981). Similarly, Na^+ concentrations below 100 mM increase the apparent dissociation constant (= weaken the binding) of specifically bound phlorizin by 15–300 times in vesicle studies (Chesney et al., 1974; Turner & Silverman, 1981). Thus, our results are consistent with the known properties of the glucose transporter (Semenza et al., 1984).

Multiple Carriers

If there were only a single type of glucose carrier, the Scatchard plot of Fig. 4 would be a straight line. It is not for the intact mucosa (our results) nor for brush-border membrane vesicles from the small intestine of rabbits (Tannenbaum et al., 1977). Studies by Turner and Moran (1982*a*,*b*) suggest two populations of Na^+/D-glucose cotransporters in kidney: a system with high affinity for phlorizin (K_d = 0.18 μM), low affinity (K_m = 6.0 mM) and high capacity (V_{max} = 10 nmol/min, mg protein) for glucose, 1 : 1 Na^+/glucose stoichiometry, and prevailing in the renal outer cortex; and another system with low affinity for phlorizin (K_d = 100 μM), high affinity (K_m = 0.4 mM) and low capacity (V_{max} = 4 nmol/min, mg protein) for glucose, 2 : 1 Na^+/glucose stoichiometry, and prevailing in the renal outer medula. Our

estimate of phlorizin affinity (K_d) for our intestinal site presumed to have the higher affinity is 0.16–0.17 μM (this paper, mice on a chow diet) or 0.06–0.08 μM (Ferraris & Diamond, 1986, mice on other diets), similar to Turner's and Moran's estimate for their high-affinity site in kidney (K_d = 0.1 μM) and far below their estimate for their low-affinity site (100 μM).

Our measurements of V_{max} for glucose transport in intestine (Ferraris & Diamond, 1986) are performed at a glucose concentration of 50 mM, far above the glucose K_m (6 or 0.4 mM) of either of Turner's and Moran's systems. Both systems would therefore be saturated under conditions of our V_{max} determinations, and V_{max} should mainly reflect the first system, which has the higher capacity for glucose. The apparent K_m of the first system in kidney (6 mM) also agrees with the apparent K_m of glucose uptake in mouse intestine (6.2 mM: Karasov & Diamond, 1983*b*). Thus, the first system is probably the one responsible for most glucose transport in our experiments. In brush-border membrane vesicles from rabbit jejunum, Kaunitz and Wright (1984) also observed the existence of a system with high affinity (K_m = 0.03 mM) and low capacity for glucose, as well as the major system with high capacity but lower affinity (K_m = 2 mM) for glucose.

This is not to deny the possibility that the low-capacity system could be more important in experiments with lower glucose concentrations or with mouse ileum rather than jejunum. For example, Freeman and Quamme (1986) obtained kinetic evidence for the presence of two Na^+-dependent glucose cotransporters in brush-border membrane vesicles from the intestine of young rats. The cotransporter located only in the jejunum of young rats (but extending into the ileum of adults) is characterized by a high capacity and low affinity for glucose, while another located throughout the jejunum and ileum is characterized by low capacity and high affinity.

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