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Use of Phlorizin Binding to Demonstrate Induction of Intestinal Glucose Transporters

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Summary. We used specific binding of phlorizin to the intact intestinal mucosa in order to measure glucose transport site density in intestines of mice fed a high-carbohydrate or no-carbohydrate diet. Nonspecific binding varied with intestinal position but showed only modest dependence on diet. Specific binding to glucose transporters was 1.9 times greater in jejunum of high-carbohydrate mice than of no-carbohydrate mice; this ratio was the same as the ratio for V_{max} values of active D-glucose uptake between the two diet groups. The gradient in specific binding of phlorizin along the intestine paralleled the gradient in V_{max} of glucose transport. These results directly demonstrate that the increase in intestinal glucose transport caused by a high-carbohydrate diet is due to induction of glucose transporters. They also indicate that the normal positional gradient in glucose transport along the intestine arises from a gradient in transporters, induced by the normal gradient in luminal glucose concentration.

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

Introduction

Intestinal active uptake of monosaccharides is subject to adaptive regulation under numerous physiological and pathological conditions (Karasov & Diamond, 1983, 1986). The best studied of these responses is that to changes in dietary carbohydrate levels (Hahn & Koldovsky, 1966; Bode, Eisenhardt, Haberich & Bode, 1981; Scharrer et al., 1981; Karasov, Pond, Solberg & Diamond, 1983; Diamond & Karasov, 1984). When dietary carbohydrate is increased or decreased, duodenal and jejunal D-glucose uptake reversibly increases in ca. 1 day or decreases in several days, respectively. Kinetic analysis of glucose uptake shows that this response involves a change in V_{max}, which is twofold higher in mice on high-carbohydrate diets than on carbohydrate-free diets. [An associated apparent change in the Michaelis-Menten constant K_m is thought to result from the V_{max} change as an unstirred-layer artifact (Karasov, Buddington & Diamond, 1986).] This response of V_{max} for active D-glucose uptake is specific: passive permeability to L-glucose remains unchanged, and amino acid uptake varies independently in response to dietary protein levels. Hence it has been postulated that the responses of D-glucose uptake involves induction of glucose transporters by dietary substrate levels. Supporting this interpretation is the observation that a similar response of D-glucose uptake to changes in blood glucose concentration is suppressed by an inhibitor of protein synthesis (Csaky & Fischer, 1981).

Possibly related to this response to dietary carbohydrate is the normal positional gradient of D-glucose uptake rates along the intestine. Uptake rises slightly from the duodenum to a peak in the jejunum, then declines steeply towards the ileum. This gradient parallels the normal gradient in luminal glucose concentration: glucose rises from the duodenum to jejunum (because pancreatic and intestinal amylases entering the duodenal lumen hydrolyze glycogen and starch to glucose), then declines from jejunum to ileum (because of glucose absorption). Hence it is tempting to assume that the "standing gradient" of glucose uptake along the intestine also involves induction of glucose transporters, whose activity at any particular site is set by the usual luminal glucose concentrations at that site (Karasov & Diamond, 1983). In agreement with this interpretation, the gradient in glucose uptake disappears in mice on a carbohydrate-free diet: duodenal and jejunal uptakes decline to the low level normally observed in the ileum (Diamond & Karasov, 1984).

Other conditions marked by glucose uptake responses suggestive of induction include starvation, diabetes, and total parenteral feeding. However, in none of these cases has a change in glucose transport site density been directly studied. This gap in our knowledge contrasts with our presently much deeper understanding of regulation of intestinal hydrolases, whose response to dietary substrates has been studied at the molecular level (Reischenauer & Gray, 1985). As a next step in understanding regula-

tion of intestinal glucose transport, we need to be able to measure glucose transport site density.

The preceding paper (Ferraris & Diamond, 1986) described a method that employs selective binding of phlorizin, a competitive inhibitor of D-glucose uptake at the intestinal brush border membrane, to measure glucose transport site density in the intact mucosa. Since the method involves the same everted sleeve preparation of intestine used for measuring D-glucose uptake, one can compare uptake and site density without concern for possible differences related to different preparations (e.g., vesicles *vs.* intact mucosa). In the present paper we use this method to ask two questions:

1. Does the change in V_{max} for active D-glucose uptake in response to altered dietary carbohydrate levels really reflect proportionate changes in glucose transport site density?
2. Does the positional gradient of uptake similarly reflect a positional gradient of site density?

Materials and Methods

Animals and Diets

Sixteen adult male white Swiss Webster mice (30-40 g) were maintained as described in the preceding paper, except that two alternative isocaloric diets were used: one diet with high carbohydrate (55% sucrose) and moderate protein (15% casein) levels, the other diet with high protein levels (70% casein) and no carbohydrate, eight mice maintained on each diet. The diets were otherwise identical to each other and were the same as the diets used previously in this laboratory to study changes in glucose uptake (*see* Diamond & Karasov, 1984, for detailed composition of the diets). The diets were supplied in pellet form by ICN Nutritional Biochemicals. Food consumption was estimated by weighing the remaining pellets twice a week; the mice did not scatter uneaten food. Mice were maintained on one or the other diet for 14 days before sacrifice. We always sacrificed mice from both diets on the same day. All measurements reported in this paper were obtained from the same two groups of eight mice on each diet.

Glucose Uptake Measurements

D-glucose uptake by everted sleeves of intestine was measured by the procedure of Karasov and Diamond (1983), except that solution pH was 7.0 (the pH used for phlorizin binding studies) rather than 7.4. Briefly, a 1-cm sleeve was preincubated for 5 min at 37°C in normal Ringer (composition in Ferraris & Diamond, 1986), then incubated for 1 min at 37°C in a stirred flat-bottomed test tube containing Ringer + 50 mM D-glucose (isosmotically replacing NaCl), ^{14}C D-glucose, and ^{3}H L-glucose. L-glucose served to correct simultaneously for passive uptake and for D-glucose in adherent fluid, yielding active uptake of D-glucose (Karasov & Diamond, 1983). At the end of the incubation the tissue was rinsed for 20 sec in 25 ml ice-cold Ringer stirred at 1000 rpm, then counted as described in the preceding paper. D-glucose uptake was measured in one sleeve from the upper jejunum (adjacent to duodeno-jejunal boundary) and one sleeve from the lower jejunum (near jejuno-ileal boundary) of each mouse. One tissue immediately adjacent to each tissue used for glucose uptake measurements was preincubated for 5 min at 37°C in Ringer, then used to measure the dry wt/wet wt ratio, which proved to be the same in the upper and lower jejunum.

Phlorizin Binding Measurements

Based on the results of the preceding paper, we incubated sleeves for 120 sec at 37°C in ^{3}H phlorizin-containing Ringer + 50 mM mannitol or Ringer + 50 mM D-glucose, rinsed for 20 sec in ice cold Ringer + 50 mM mannitol, and calculated specific phlorizin binding as the difference between binding in mannitol and in glucose. Tissues with visible Peyer's patches were discarded during the dissection.

In experiments on the effect of dietary carbohydrate levels on phlorizin binding, we took eight 1-cm sleeves from the mid-jejunum of each mouse and measured binding at four phlorizin concentrations (0.005, 0.04, 0.32, and 1.28 μM), one sleeve in each of the two incubation solutions at each phlorizin concentration. To eliminate effects of variation due to sleeve position within the jejenum, we chose four pairs of tissues (one tissue each for incubation in mannitol and in glucose) from various jejunal positions and randomly assigned the four phlorizin concentrations to the four tissue pairs. The coefficient of variation of phlorizin binding in adjacent sleeves of the same animal was 5.0 $\pm$ 3.5% (n = 4).

In experiments on the effect of intestinal position on phlorizin binding, we took six 1-cm sleeves from the duodenum (defined as anterior 6 cm of intestine) and six from the ileum (defined as posterior 6 cm of intestine) of mice fed the high-carbohydrate diet. The length of mouse duodenum or ileum allowed us to use only six sleeves (three pairs) and three phlorizin concentrations (0.005, 0.04, and 0.32 μM).

Density of equilibrium phlorizin binding to the specific high-affinity sites was calculated from Scatchard plots of amount bound/concentration *vs.* amount bound (*cf.* Fig. 4 of preceding paper). From such plots one extracts an estimate of binding site density for one region of intestine (duodenum, jejunum, or ileum), based on the measurements at different phlorizin concentrations in three or four adjacent pairs of sleeves from the region. Nonspecific binding normalized to tissue weight was found not to vary with position along the intestine. However, specific binding normalized to tissue weight decreased from proximally to distally, by 5.0 $\pm$ 3.4% (n = 4) per cm. Since the sleeves were 1 cm long and since we prepared 6-8 sleeves per region, this gradient means that specific binding in the most distal sleeve of a region would be about 30% less than specific binding measured in the most proximal sleeve in the same incubation solution. Hence we normalized all values of specific binding to the most proximal position in the region, on the assumption of a gradient of 5%/cm.

Statistical analyses were performed as in the preceding paper.

Results

Effect of Diet on Body Weight, Food Consumption, and Gut Length and Mass

The high-carbohydrate mice exceeded no-carbohydrate mice slightly but significantly in food consumption, and slightly but not significantly in weight gain (Table 1). Diet had no effect on intesti-

Table 1. Effect of diet on body weight, food consumption and gut mass

	Diet		P value
	No Carbohydrate	High Carbohydrate	
Body weight			
Initial (g)	38.2 ± 1.6	38.5 ± 0.8	0.86
Final (g)	38.1 ± 1.0	40.3 ± 0.7	0.09
Gain (g/day)	−0.01 ± 0.08	0.13 ± 0.05	0.12
Gain (%/day)	0.00 ± 0.20	0.34 ± 0.11	0.16
Food Consumption			
Daily consumption (g/mouse)	3.4 ± 0.14	4.4 ± 0.27	0.004*
Daily consumption per unit weight	0.09 ± 0.003	0.11 ± 0.006	0.003*
Gut length and mass			
Total length (cm)	45.8 ± 1.3	46.6 ± 1.5	0.69
Weight per unit length (mg/cm)			
a) duodenum	—	59.7 ± 2.3	
b) jejunum	43.2 ± 2.8	42.0 ± 3.3	0.79
c) ileum	—	17.2 ± 1.0	
Dry weight (%)	15.1 ± 0.7	14.8 ± 0.8	0.76
Gut length/body weight (cm/g)	1.21 ± 0.04	1.18 ± 0.04	0.72

[a] Final weights were measured after 2 weeks on the indicated diet. Percent dry wt is an average of values for upper and lower jejunum, which did not differ significantly. Right-most column gives significance level for difference between values on the two diets; asterisks denote a significant difference at the $P < 0.05$ level. Sample size was eight mice for each diet. Values of weight per unit length of intestine, and of percent dry wt, are based on two 1-cm sleeves per region and/or per mouse.

Table 2. Effect of diet and intestinal region on the affinity constant and density of phlorizin binding sites

	Diet			
	No carbohydrate	High carbohydrate		
	Jejunum	Duodenum	Jejunum	Ileum
Density (pmol/mg)	0.278 ± 0.073 (21)[a]	0.604 ± 0.159 (15)[b]	0.554 ± 0.125 (19)[b]	0.165 ± 0.040 (20)[a]
Apparent K_d (nM)	74 ± 35[c]	77 ± 37[c]	65 ± 28[c]	68 ± 29[c]

The density of phlorizin binding sites was calculated as the X intercept ± estimated SE of X intercept (total number of points of Scatchard plots). The apparent K_d was calculated from the slope ± SE of the slope. Results are derived from eight mice on each diet. Slopes differed from 0 at significance levels of $P < 0.05$ or better. Numbers that share the same superscript in the same row are statistically not different ($P > 0.05$).

nal length, jejunal wet wt per cm, intestinal dry wt/wet wt, or intestinal length normalized to body weight. Diamond and Karasov (1984) obtained similar results and also showed that passive glucose permeability and nominal and villus surface area are the same in high-carbohydrate as no-carbohydrate mice.

Effect of Diet and Intestinal Position on Phlorizin Binding

The density of high-affinity, glucose-protectable binding sites was 1.9-fold higher in jejunum of high-carbohydrate than no-carbohydrate mice (Table 2). In high-carbohydrate mice site density did not differ

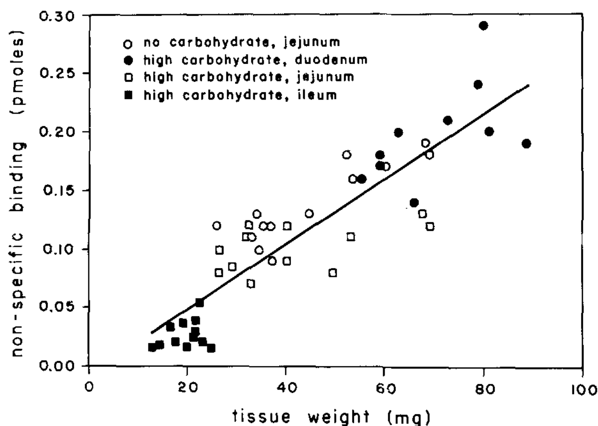

Fig. 1. Nonspecific binding of ^{3}H-phlorizin at 0.005 μM as a function of tissue weight. Tissues were chosen at random from three intestinal regions (●, duodenum; □, jejunum; ■, ileum) of mice fed high carbohydrate, and from the jejenum of mice fed no carbohydrate (○). The slopes of nonspecific phlorizin binding against weight of tissue were not significantly different ($P > 0.20$) among the four segments, hence data from all groups were pooled. The resulting regression coefficient is 2.8 ± 0.2 (49) fmol/mg when the bathing phlorizin concentration is 0.005 μM (*see* text for regression coefficient of each tissue group). There was a significant difference in the individual Y intercepts ($P < 0.05$), but the Y intercept of the pooled data (−7.0 ± 10.6 fmol) did not differ from 0 ($P > 0.60$)

significantly ($P > 0.25$) between duodenum and jejunum but was 3.3-fold higher ($P < 0.001$) in jejunum than in ileum, 3.7-fold higher ($P < 0.01$) in duodenum than in ileum. The apparent dissociation constant for phlorizin binding, K_d, did not differ between duodenum, jejunum, and ileum of high-carbohydrate mice, nor between jejunum of high-carbohydrate and no-carbohydrate mice (Table 2). Our values for site density and K_d of jejunum of mice on a high-carbohydrate diet are somewhat lower than the corresponding values reported in the previous paper (Ferraris & Diamond, 1986) for jejunum of mice on a chow diet, presumably because of the different diets, batches of mice, or phlorizin concentrations studied.

Effect of Diet and Intestinal Position on Active D-Glucose Uptake

We measured active D-glucose uptake at 50 mM in jejunum of high-carbohydrate and no-carbohydrate mice. Uptake at 50 mM approximates the V_{max} (Diamond & Karasov, 1984). Uptake did not differ significantly ($P > 0.10$) between upper and lower jejunum of no-carbohydrate mice but was higher ($P < 0.01$) in upper (12.4 ± 1.3 nmol/mg wet wt · min (n = 7)) than in lower (5.7 ± 0.5 (7)) jejunum of high-carbohydrate mice. Thus, a positional gradient in jejunal uptake is present in our high-carbohydrate but not no-carbohydrate mice, in accord with previous studies of the positional gradient along the length of the small intestine (Diamond & Karasov, 1984, Figs. 2 and 6).

For comparison with our phlorizin binding measurements, which were taken from upper to lower jejunum, we averaged D-glucose uptake values in upper and lower jejunal sleeves: 9.1 ± 1.1 (14) and 4.8 ± 0.5 (14) nmol/mg · min in high-carbohydrate and no-carbohydrate mice, respectively. Thus, uptake on the high-carbohydrate diet exceeds that on the no-carbohydrate diet by a factor of 1.9. These values are very close to those obtained by Diamond and Karasov (1984) for mice on these same two diets (*see* Fig. 2).

Effect of Diet and Intestinal Position on Nonspecific Phlorizin Binding

Figure 1 depicts nonspecific phlorizin binding at a phlorizin concentration of 0.005 μM, as a function of tissue weight. Jejunal tissues from high- and no-carbohydrate mice yielded similar ($P > 0.25$) slopes: 1.0 ± 0.5 (13) and 1.5 ± 0.4 (13) fmol/mg, respectively. Slopes for duodenal tissues (2.2 ± 1.0 (13) fmol/mg) and ileal tissues (0.9 ± 1.0, no significant regression, because abscissa values show little spread) from high-carbohydrate mice do not differ significantly ($P > 0.1$) from the jejunal slope for the same mice because of the large standard errors. However, the slope of the pooled data (2.8 ± 0.2 (49) fmol/mg) is higher than these slopes for each of the subsets, because nonspecific binding per mg is higher in the duodenum (high-abscissa values in Fig. 1) than in ileum (low-abscissa values in Fig. 1).

These conclusions are reinforced by plotting nonspecific binding against phlorizin concentration. The relation is linear (correlation coefficient ≥ 0.9, significant at $P < 0.001$) in all four cases (jejunal tissues from high- and no-carbohydrate mice, duodenal and ileal tissues from high-carbohydrate mice), suggesting that nonspecific binding involves nonsaturable sites present in excess for the phlorizin concentrations we used. (In contrast, specific binding increases nonlinearly with concentration and involves a limited number of sites.) The slopes for jejunal tissues of no-carbohydrate (0.80 ± 0.06 (28) pmol/mg, μM) and high-carbohydrate (0.63 ± 0.06 (25) pmole/mg, μM) mice do not differ ($P > 0.20$, two-tailed t test). Slopes for duodenal (0.50 ± 0.17 (15), jejunal (0.63 ± 0.06 (n = 25)), and ileal (0.22 ± 0.14 (n = 20)) tissues of high-carbohydrate mice differ slightly, but only the comparison for je-

junum *vs.* ileum approaches significance ($P \sim 0.09$). These positional differences in non-specific binding are not surprising: non-specific binding is thought to involve membrane lipids and proteins (other than the glucose transporter), both of which are likely to vary with position.

Discussion

Figure 2 plots specific phlorizin binding site density against V_{max} of active D-glucose transport, as a function of diet for jejunal sleeves, and as a function of intestinal position on the high-carbohydrate diet. Changes in site density and in V_{max} are closely correlated ($r = 0.98$). The Y intercept of 0.57 ± 1.18 (6) nmol/mg · min does not differ significantly from zero ($P > 0.4$), suggesting that variations in density of the high-affinity phlorizin binding sites underlie most of these variations in glucose uptake by mouse intestine. If the Y intercept is real, it might represent glucose transport by the low-affinity phlorizin binding sites (Ferraris & Diamond, 1986), but a test of this possibility will require more accurate measurements of this binding than we were able to achieve.

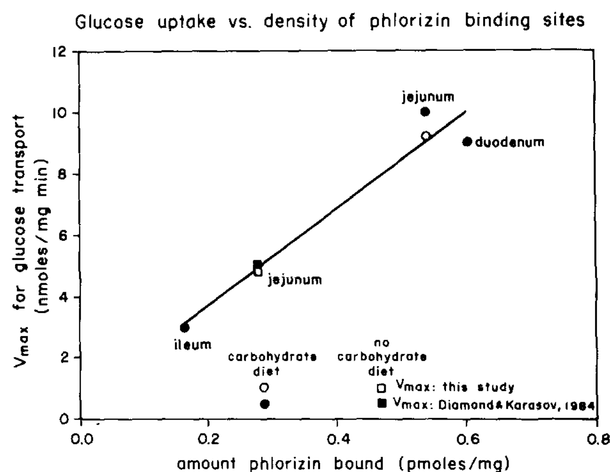

Fig. 2. Active uptake of D-glucose at 50 mM, as a function of the density of glucose-protectable, high-affinity phlorizin binding sites. Open circles or squares: glucose uptake and phlorizin binding measured simultaneously in adjacent sleeves. Values for glucose uptake are given in the text. Closed points: glucose uptake values taken from Diamond and Karasov (1984), who measured glucose uptake as a function of distance from pylorus. Duodenal and ileal values are from uptake rates in segments taken 1 and 54 cm from the pylorous, respectively, while jejunal values are the average of uptake values in segments 12, 28, and 40 cm from the pylorus. The regression is highly significant ($P < 0.001$), with a correlation coefficient of 0.98. The slope, which represents turnover number, is $15{,}600 \pm 1{,}800$ (min^{-1}), while the Y intercept is 0.57 ± 1.18 nmol mg^{-1} min^{-1} (not significantly different from 0: $P > 0.4$)

Figure 2 yields four further conclusions:

1. *Dietary effect on glucose transport.* Switching from a no-carbohydrate to a high-carbohydrate diet increases the V_{max} of active D-glucose transport by 1.9-fold (this study; Diamond & Karasov (1984) obtained the same factor). This factor is the same as the proportional increase in specific phlorizin binding, by 1.9-fold. Thus, the postulate of previous authors that dietary carbohydrate enhances glucose transport rates by inducing glucose transporters (or by reducing their rate of degradation) is directly confirmed. Our results mean that the increase in transporter number fully accounts for the increase in transport rate; no other effect of diet need be involved. Amsler and Cook (1985) recently reached the same conclusion for induction of glucose transporters in LLC-PK cells in culture.

2. *The positional gradient of glucose transport.* Active uptake of 50 mM D-glucose in proximal intestine exceeds ileal uptake by 3-6 times, whether uptake is normalized to mg wet wt of intestinal tissue, mg scrapable mucosa, cm^2 nominal surface area, cm^2 villus surface area (Diamond & Karasov, 1984), or cm^2 microvillus surface area (P. Lee & J.M. Diamond, *unpublished observations*). Thus, the gradient in mucosal mass (mg per cm of intestine), which decreases from the duodenum towards the ileum, contributes to the gradient in glucose transport expressed per cm of intestine, but fails to account for the gradient in glucose transport expressed per quantity of intestinal tissue. However, the density of specific phlorizin binding sites in proximal intestine is 3.7 times that in ileum. Thus, the positional gradient in glucose transport per quantity of tissue is due to the gradient in glucose transport site density, as previously postulated (Diamond & Karasov, 1984). The reason why the ileum has low glucose transport activity, even on high-carbohydrate diets, is that most glucose is absorbed in the duodenum and jejunum so that little remains to induce glucose transport in the ileum. However, glucose does reach the ileum and ileal glucose transport does become enhanced on high-glucose diets sufficient to saturate duodenal and jejunal transport capacity (Nunn & Ellert, 1967; Bode et al., 1981), or when ileal segments are transplanted more proximally (Dowling, 1973), or when the jejunum is excised (Dowling & Booth, 1967; Urban & Haley, 1978).

3. *Nonrepressible glucose transporters.* While many of the glucose transporters become repressed in the absence of dietary substrates, considerable transport capacity remains—even in the ileum of no-carbohydrate mice (Diamond & Karasov, 1984), where luminal glucose should be minimal or zero.

These residual transporters may function to reabsorb any glucose that diffuses from the bloodstream to the intestinal lumen under conditions where little glucose enters the lumen via the diet (Silk & Dawson, 1979; Naftalin & Kleinzeller, 1981). It will be interesting to find the molecular basis for this irreducible minimum of transport capacity. Is it programmed into the developing enterocytes as they leave the intestinal crypts?

4. *Turnover number and site number.* The slope of Fig. 2, 16,000/min^{-1}, equals the turnover number (for one phlorizin binding site) of the mouse intestinal glucose transport at 37°C. Stirling (1967) estimated a turnover number of 1,260/min (temperature not stated) for hamster intestinal glucose transporter, but this value is probably low because of inadequate compensation for nonspecific binding and consequent overestimation of specific binding site density. Wright and Peerce (1985) recently estimated turnover rate of 360/min at 22°C for the glucose transporter in brush border membrane vesicles of rabbit small intestine, while Amsler and Cook (1985) estimated 10,200/min at 37°C in LLC-PK_1 cells.

Stirling (1967) and Diedrich (1968) also estimated, again without correction for nonspecific binding, the number of transporters per cell of hamster intestine as $2.6 \times 10^6 - 4 \times 10^8$, while Wright and Peerce (1985) gave an estimate of 10^5-10^6 transporters per cell of rabbit intestine. From our data we estimate about 10^6-10^7 transporters per cell in mice on a high-carbohydrate diet, and about 10^{14}-10^{15} transporters for the whole length of the mouse small intestine.

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