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Ontogeny of Na^+/D-glucose cotransport in guinea-pig jejunal vesicles: only one system is involved at both 20°C and 35°C

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The kinetic parameters of Na^+/D-glucose cotransport were examined in fetal, newborn and adult guinea-pig jejunal brush-border membrane vesicles using a displacement curve and non-linear regression procedure. Our data indicated the presence of a single system with a K_m of 0.34 ± 0.04 mM at both 20°C and 35°C. V_{max} was increased by about 4-fold when the kinetic experiments were performed at 35°C. Since our results were not in agreement with the findings of Brot-Laroche et al. (J. Biol. Chem. (1986) 261, 6168–6176) which indicated the existence of a distinct D-glucose transport system in the adult guinea-pig jejunum at 35°C, we verified the influence of their experimental conditions on initial rate uptake measurements. In the presence of D-sorbitol instead of D-mannitol in the transport media, 70% inhibition of D-glucose uptake was observed, an effect which was attributable to contamination of sorbitol preparations by D-glucose. After removal of glucose contamination D-sorbitol did not significantly reduce the initial rate of D-glucose transport. These results led us to conclude the existence of a single D-glucose transport system in the guinea-pig small intestine and to stress the choice of experimental conditions as being crucial for an accurate estimation of kinetic parameters.

Introduction

Numerous studies have been devoted to the characterization of D-glucose transport in the adult small intestine (reviewed in Refs. 1–3) but data on the ontogeny of sugar transport systems in animal models are scarse [4]. Apart from a few experiments performed on brush-border membrane vesicles (BBMV) isolated from rat [5–7], sheep [8] and chick [9] small intestines, intestinal segments have been used to measure the transepithelial transfer or tissue accumulation of substrates [10–17]. These studies have generated some information on sugar uptake capacities along the length of the small intestine as a function of developmental age, but kinetic parameters of D-glucose transport cannot be precisely evaluated under such experimental conditions (reviewed in Ref. 4).

The existence of two distinct carriers with different affinities for D-glucose along the length of the small intestine has been proposed in many adult species [2,3], although this issue is still controversial [2]. In our studies concerning Na^+/D-glucose cotransport in the human small intestine, the presence of two carriers has been documented in the fetus [18,19], whereas a single system has been found in adults [20]. These recent findings raised many questions about the normal development of transport functions in the small intestine and the regulatory mechanisms prevailing during ontogeny of this organ. Obviously, the question of when and how these modifications of transport functions occur cannot be answered for the human small intestine since tissue availability is limited and experimental conditions are restricted.

Considering these problems, we have attempted to find an animal model which corresponds to human development. In this regard, the guinea-pig appears to be very interesting. Its gestational period is quite long (60–72 days) compared to other rodents, and guinea-pig pups are well developed at birth, being able to ingest solid food from day 1. Furthermore, they express transporters for sugars and amino acids at the beginning of the second half of gestation [12], as does the human fetus [21]. According to Brot-Laroche et al. [22], two different D-glucose carriers are expressed in the adult guinea-pig: (i) a low-temperature-sensitive system with

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a high affinity for D-glucose; and (ii) a high-temperature-sensitive system with a low affinity for D-glucose. This second system, measured at 35°C, is stimulated by semi-starvation [23]. Even if no data on these systems in fetal and neonatal development are actually available, the guinea-pig seems to be suitable for the study of the ontogeny of glucose carriers and for the investigation of factors involved in the regulation of transport functions during development.

Taking advantage of a state-of-the-art fast sampling, rapid filtration apparatus (FSRFA) [24] and non-linear regression analysis of data plotted on a displacement curve, as proposed recently [20], the present study was first aimed at determining the kinetic characteristics of D-glucose transporter(s) during the normal development of guinea-pig small intestine. Since our results were not in agreement with previous findings in the adult guinea-pig small intestine [22], we focused our efforts on comparing the conditions under which the kinetic experiments were performed.

Materials and Methods

Tissues

Either pregnant Hartley guinea-pigs of known gestational age, newborns (10- and 15-day-old) and adult (350–400 g) animals were obtained from Charles River Canada (St. Constant, Québec). They were fed vitamin C-containing pellets and had free access to water before being killed by decapitation. The fetuses were taken at 55–60 days of gestation.

Preparation of brush-border membrane vesicles

Intestinal tissues were processed as follows: the proximal part of the small intestine was removed, washed with ice-cold saline solution, and the mucosa was scraped with a spatula onto a cold glass plate. In the case of fetuses, whole segments were used for membrane preparation. On average, 10 tissues were pooled for each transport experiment. Brush-border membranes were purified by $MgCl_2$ [25] or $CaCl_2$ [26] precipitation and vesicles prepared as described previously [18]. The pellets obtained after the second centrifugation step (P_2 fractions) were resuspended in a minimum volume of 50 mM Tris-Hepes (TH) buffer (pH 7.5), 0.1 mM $MgSO_4$, 250 mM KCl and proteinase inhibitors, as outlined elsewhere [20], and frozen in liquid nitrogen. In another set of experiments, vesicles were resuspended in 10 mM Hepes, 7 mM maleic acid, 7 mM butylamine buffer (HMBA) (pH 7.4), and 500 mM D-sorbitol as described earlier [22]. On the day of the experiment, the final vesicle pellet (P_4) was prepared, resuspended in the medium required, divided into 25-μl aliquots and frozen in liquid nitrogen until the time of assay to prevent instability and loss of transport activity during the kinetic experiments [27]. Based on sucrase activity, an enrichment factor of 10–12-fold over the homogenate was routinely obtained.

Transport studies

BBMV were resuspended to a final concentration of 15–25 mg/ml in the final resuspension buffer and aliquots of 20 μl/0.5 ml incubation medium were used. The exact composition of the resuspension and incubation media is given in the legends to the figures and tables. Kinetic experiments were performed under 0 mV voltage-clamped conditions, using K^+-valinomycin and 0.5 mM amiloride in all uptake media [20] except when stated otherwise in the legends to both figures and tables. In another set of experiments, initial rates of D-glucose uptake were determined in the presence of a valinomycin-induced inside-negative membrane K^+-diffusion potential.

D-[^{3}H]Glucose uptake was measured according to the rapid filtration technique of Hopfer et al. [28], with a fast sampling, rapid filtration apparatus (FSRFA) as described recently [24] which allows sampling of up to 18 aliquots from the same incubation mixture over a very short time period. Each aliquot was injected in 1 ml ice-cold stop solution containing 1 mM phlorizin, filtered through 0.65 μm nitrogen cellulose (Micro Filtration System, Dublin, CA) and washed three times with 1 ml stop solution. The filters were then dissolved in 5 ml BetaBlend (ICN Radiochemicals, Irvine, CA) and radioactivity was determined with a Minaxi Tri-Carb Series 4000 scintillation counter (United Technologies Packard, Downers Grove, IL).

Data analysis

Transport data were expressed as pmoles solute uptake/s per mg protein. Initial rates of D-glucose uptake were estimated at both 20°C and 35°C by using nine points over 4.5 s and either linear [20] or polynomial [29] regression analysis. The kinetic parameters of D-glucose uptake (values $\pm$S.D. of regression) were determined by non-linear regression analysis, as discussed elsewhere [30], and with a displacement curve, as proposed recently [20]. Different model equations corresponding to either one or two Michaelian saturating components working in the presence or absence of a nonspecific component were tested for each set of kinetic data, as done previously [20]. Eadie-Hofstee linear transformation of the data are shown in the figure insets.

The Hill equation describing (n sites) model was also applied to each set of kinetic data. In such case, the Hill number (n_H), representing the number of substrate binding sites, will assume any value from 0 to n that verifies $0 < n_H \leq n$. However, in the case of $n_H = 1$, the Hill equation reduces to the Michaelis-

Menten equation, thus supporting the presence of a single system [31,32,36].

The inhibition curve was fitted according to the competitive inhibition equation [31]. In all cases, the same software was used (Enzfitter, Robin J. Leatherbarrow, Copyright 1987, Elsevier-Biosoft) on a IBM PC compatible microcomputer.

Biochemical assays

Sucrase activity (EC 3.2.1.48) and D-glucose concentration were estimated by continuous spectrophotometric assay based on the hexokinase reaction proposed by Kunst et al. [33]. The D-glucose concentration in both mannitol and sorbitol solutions was also evaluated by high performance liquid chromatography (HPLC) performed by a private laboratory (Analex, Laval, Québec, Canada). Proteins were measured with the BCA Protein Assay Reagent (Pierce, Rockford, IL), using bovine serum albumin as standard. Enzymatic transformation of glucose contaminants in sorbitol was performed after two elutions with immobilized glucose oxydase in a compact reaction column (F7m column containing 250 units of enzyme, Mo Bi Tec, Göttingen, Germany). The reaction was at room temperature at pH 7.5. The end products of the glucose oxidase reaction, namely D-gluconic acid and D-glucono-δ-lactone, were measured with a UV-method reaction kit (Boehringer Mannheim, Indianapolis, IN). The removal of these end products was done by a single elution on a DEAE-Sephacel ion exchanger column (Pharmacia, Uppsala, Sweden). Sorbitol concentration was determined by the sorbitol dehydrogenase reaction method of Bergmeyer et al. [34].

Chemicals

D-[1(n)-^{3}H]Glucose (15.5 Ci/mmol) was purchased from New England Nuclear (Mississauga, Ont., Canada). D-Sorbitol was obtained from three different sources, namely Sigma (St. Louis, MO), Aldrich (Milwaukee, WI) and Fisher Scientific (Nepean, Ont., Canada). D-Mannitol, amiloride hydrochloride and valinomycin were from Sigma. Phlorizin was from Aldrich. All salts and chemicals used for buffer preparation were of the highest purity available.

Results

Stability of BBMV

The stability of the vesicle preparations was evaluated over a 4-h period by measuring the initial rate of 4 μM D-glucose uptake (tracer concentration) every 2 h. With vesicles kept on ice, large variations in the initial

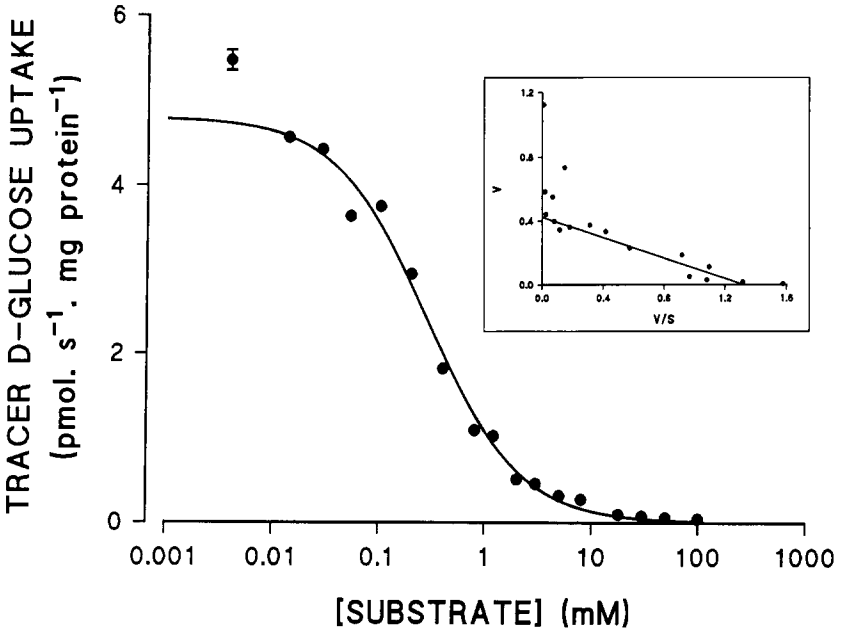

Fig. 1. Determination of kinetic parameters of D-glucose transport in adult guinea-pig jejunal BBMV at 20°C using a displacement curve and log scale for substrate concentration. The vesicles were resuspended in 50 mM Tris-Hepes (TH) buffer (pH 7.5), 0.1 mM $MgSO_4$, 100 mM KCl, 200 mM choline chloride, 125 mM D-mannitol and 5 μM valinomycin. Final concentrations in the incubation media were: 50 mM TH buffer (pH 7.5), 0.1 mM $MgSO_4$, 100 mM KCl, 192 mM NaCl, 8 mM choline chloride, 0.5 mM amiloride, 4 μM D-[^{3}H]glucose, and increasing amounts of unlabelled D-glucose (0 to 100 mM). Osmolarity of the incubation media was maintained with D-mannitol. BBMVs were prepared from a pool of eight guinea-pig jejunum. Initial rates of uptake were determined by linear regression analysis of the uptake time curve, using nine points over 4.5 s (slopes $\pm$ S.D. of regression). Non-linear regression analysis of the displacement curve gave K_m and V_{max} values of 0.32 ± 0.03 mM and 425 ± 22 pmol/s per mg protein, respectively (see Table I). Except for the lower substrate concentration, the S.D. of regression on the initial uptake rate measurements was smaller than the symbols used. The Eadie-Hofstee plot of data covering the entire range of significant substrate concentrations is shown in the inset (see Materials and Methods for more details).

uptake rate were observed after 2 h and 4 h from one preparation to the next, the loss of activity ranging from 10 to 84% of the uptake rate measured with freshly prepared vesicles (data not shown). To prevent these variations, all the kinetic experiments were performed with aliquots of BBMV frozen in liquid nitrogen until the time of assay. With this procedure, no change in uptake activity was observed from the beginning (21.7 ± 0.42 pmol/s per mg protein) to the end (21.7 ± 0.52 pmol/s per mg protein) of the experimental period.

Kinetic parameters of D-glucose transport

The initial rates of tracer D-glucose uptake were measured as a function of increasing substrate concentrations (10 μM to 100 mM) in the incubation medium at both 20° (Fig. 1 and Table I) and 35°C (Fig. 2 and Table I) in fetal, newborn and adult guinea-pig jejunum. In all cases, a single system with a mean K_m of 0.34 ± 0.04 mM ($n = 10$) was found. The goodness of fit for the 1-site model was confirmed by both Eadie-Hofstee plots (insets of Figs. 1 and 2) and Hill coefficients which were not statistically different from 1 (Table I). For all series of kinetic data, the 2-site model was rejected on the basis of either divergence or negative parameter values. The V_{max} of transport increased with temperature and, at 35°C, a small non specific component was observed (Fig. 2 and Table I).

In another set of experiments, the nature of this non specific component was investigated. In these assays, performed at 35°C, we have measured: (i) tracer D-glucose (4 μM) uptake in the absence of Na^+; or, (ii) in the presence of 192 mM Na^+ and saturating concentration of unlabelled D-glucose (250 mM); and, (iii) tracer L-glucose (4 μM) uptake under Na^+ gradient condition (Fig. 3). Uptakes were measured over a 9 s period and 10 independent measurements were made under each of these three conditions. The slopes were not significantly different at the 95% confidence interval, showing that: (i) Na^+ is required for D-glucose uptake; (ii) the non-specific component measured at

TABLE I

Effects of temperature on kinetic parameters of D-glucose transport in the guinea-pig jejunum

Each value ± S.D. of regression was obtained from a different brush-border membrane preparation. K_m is expressed in mM and V_{max} in pmol/s per mg protein. Diffusion rate constants (K_d values) are expressed in pmol/mg protein at 4 μM tracer. n_H represents the Hill coefficient, as described in Materials and Methods. n.d.: non-detectable. The experimental conditions are described in the legend to Fig. 1.

	K_m	V_{max}	K_d	n_H
20°C				
Fetuses	0.37 ± 0.04	547 ± 52	n.d.	1.04 ± 0.06
Newborns	0.36 ± 0.02	241 ± 7	n.d.	0.92 ± 0.05
	0.37 ± 0.02	474 ± 17	n.d.	0.94 ± 0.04
Adults	0.34 ± 0.03	860 ± 54	n.d.	0.95 ± 0.03
	0.32 ± 0.03	425 ± 22	n.d.	0.96 ± 0.04
35°C				
Newborns	0.29 ± 0.02	2906 ± 156	0.51 ± 0.03	0.99 ± 0.02
	0.40 ± 0.03	1809 ± 107	0.11 ± 0.04	0.95 ± 0.04
Adults	0.27 ± 0.03	2060 ± 200	0.29 ± 0.04	0.93 ± 0.04
	0.35 ± 0.02	2046 ± 117	0.25 ± 0.08	1.03 ± 0.02

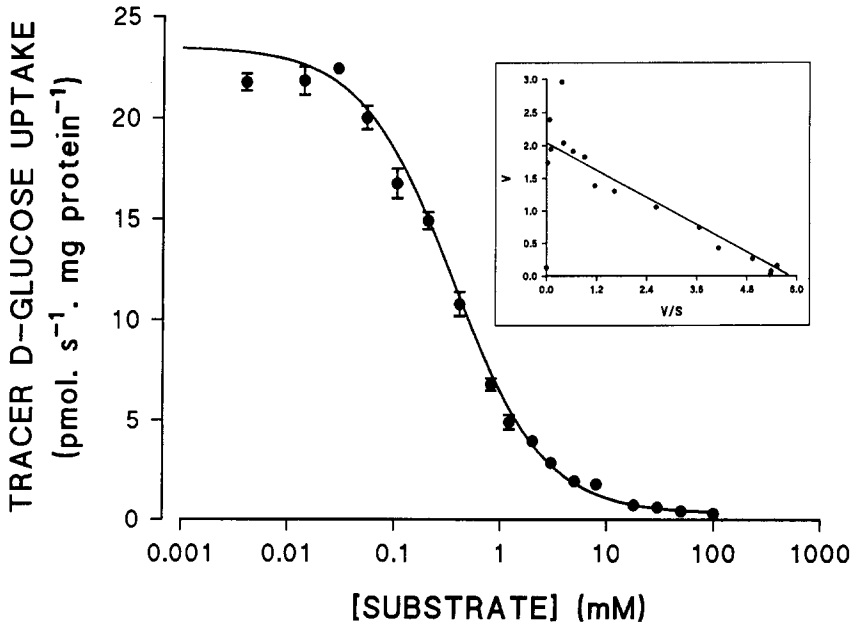

Fig. 2. Displacement curve of tracer D-glucose uptake as a function of increasing substrate concentrations in the adult guinea-pig at 35°C. The experimental conditions are described in the legend to Fig. 1. K_m, V_{max} and K_d values were 0.35 ± 0.02 mM, 2046 ± 117 pmol/s per mg protein, and 0.25 ± 0.08 pmol/s per mg protein at 4 μM tracer, respectively. The Eadie-Hofstee linear transformation plot is shown in the inset.

35°C represents passive diffusion of substrate into the vesicles; and, (iii) the estimation of diffusional component by measuring tracer uptake at saturating concentration of unlabelled substrate is adequate.

Since our results were not in agreement with those obtained by Brot-Laroche et al. [22] who reported the presence of two distinct systems for D-glucose uptake at 35°C in the adult guinea-pig jejunum, we performed kinetic studies under their experimental conditions [22]. Brush-border membranes were purified by $MgCl_2$ precipitation [25] and BBMV were resuspended in 10 mM HMBA buffer (pH 7.4) and 500 mM D-sorbitol, as described [22]. D-glucose uptake was measured in the presence of varying concentrations of unlabeled substrate (10 μM to 250 mM), and D-sorbitol was used to keep osmolarity constant. The stop solution contained: 10 mM HMBA buffer, 25 mM $MgSO_4$ and 350 mM KCl, as previously described [22]. The results obtained under these conditions are illustrated in Fig. 4. By comparing these data recorded at 35°C with our earlier results (Fig. 2), major discrepancies were noted: (i) the highest values for tracer D-glucose uptake were around 7-times lower; (ii) both K_m (0.35 ± 0.02 vs. 0.80 ± 0.10 mM) and V_{max} values (2046 ± 117 vs. 655 ± 60 pmol/s per mg protein), estimated under our (Fig. 2) and their conditions (Fig. 4), respectively, were largely different; (iii) the model equation for two sites gave the following parameter values: K_{m1}: 0.69 ± 0.08 mM; $V_{max,1}$: 562 ± 53 pmol/s per mg protein; K_{m2}: 143 ± 76 mM; $V_{max,2}$: 6744 ± 2661 pmol/s per mg protein; (iv) the model equation for two sites plus diffusion could not account for these data, S.D. values for the second site being larger than the estimated parameter and negative values for the K_d (diffusion rate constant) being obtained.

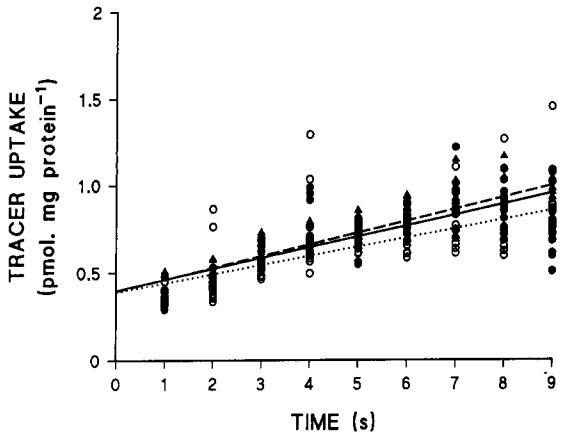

Fig. 3. Estimation of the diffusive component of D-glucose uptake. Vesicles were resuspended in: 50 mM TH buffer (pH 7.5), 0.1 mM $MgSO_4$, 100 mM KCl, 200 mM choline chloride, 300 mM mannitol and 3 μM valinomycin. The final concentrations in the uptake media were: 50 mM TH buffer (pH 7.5), 0.1 mM $MgSO_4$, 100 mM KCl, 192 mM NaCl and 8 mM choline chloride (●———●, ○·····○) or 200 mM choline chloride (▲ — — — ▲), 250 mM unlabeled D-glucose and 50 mM mannitol (●———●) or 300 mM mannitol (○·····○, ▲ — — — ▲), 3 μM valinomycin and either 4 μM D-[^{3}H]glucose (●———●, ▲ — — — ▲) or 4 μM L-[^{3}H]glucose (○·····○). Ten uptake time courses were run under each of the above experimental conditions. The lines shown are the best-fit over all the individual data points in each set of experiment. Linear regression analysis gave slope (pmol/s per mg protein) and intercept (pmol/mg protein) values (±S.D. of regression) of: 0.067 ± 0.005 and 0.394 ± 0.055 (▲ — — — ▲); 0.062 ± 0.005 and 0.400 ± 0.029 (●———●); and, 0.052 ± 0.008 and 0.388 ± 0.043 (○·····○).

Effects of experimental conditions on initial D-glucose uptake rate

To understand why different results were obtained when the experimental conditions were varied, the respective effects of either stop solution, buffers (TH and HMBA) and substituting sugars (D-mannitol and D-sorbitol) were analysed. Since brush-border membranes are usually purified using either $CaCl_2$ [26] or $MgCl_2$ [25] precipitation, both methods have also been compared. Considering the data enumerated in Table II, the following conclusions were drawn: (i) slightly higher D-glucose uptake values were obtained with $MgCl_2$- compared to $CaCl_2$-precipitated membranes; although the yield was higher with $MgCl_2$- compared to $CaCl_2$-precipited vesicles (0.74 and 0.31 mg protein, respectively, per g of mucosa), the purification factor was similar (10-fold, as estimated by sucrase activity); (ii) significant decreases of the initial D-glucose uptake rate were recorded with HMBA buffer compared to TH buffer in both vesicle preparations; (iii) the isosmolar stop solution containing phlorizin was slightly better in preventing substrate efflux from the vesicles. These results indicated that neither the stop solution nor the vesicle preparation were the main factors responsible for the observed decrease of D-glucose uptake.

Since a major difference in initial uptake rate values was observed between TH-mannitol and HMBA-sorbitol media, a cross experiment was performed to delineate the respective effects of these components. As shown in Table III, the initial rate of D-glucose uptake was largely reduced (57–66%) when sorbitol was utilized instead of mannitol but the choice of buffer was not critical. Similar decreases (mean value: $65.9 \pm 6\%$) of the initial D-glucose uptake rate were consistently recorded at both 20°C (7.6 ± 1.5 vs. 2.6 ± 1.2 pmol/s per mg protein in mannitol and sorbitol media, respectively; $n = 3$) and 35°C (18.1 ± 0.2 vs. 6.5 ± 0.2 pmol/s per mg protein, $n = 2$) either at 0 or negative membrane potentials (data not shown). Moreover, the inhibition of D-glucose uptake by D-sorbitol was concentration-dependent up to 500 mM, giving an apparent Ki value of 64 ± 15 mM (data not shown). Higher concentrations of sorbitol could not be assayed, maximal solubility being reached at 0.8 and 1 M for both mannitol and sorbitol stock solutions, respectively.

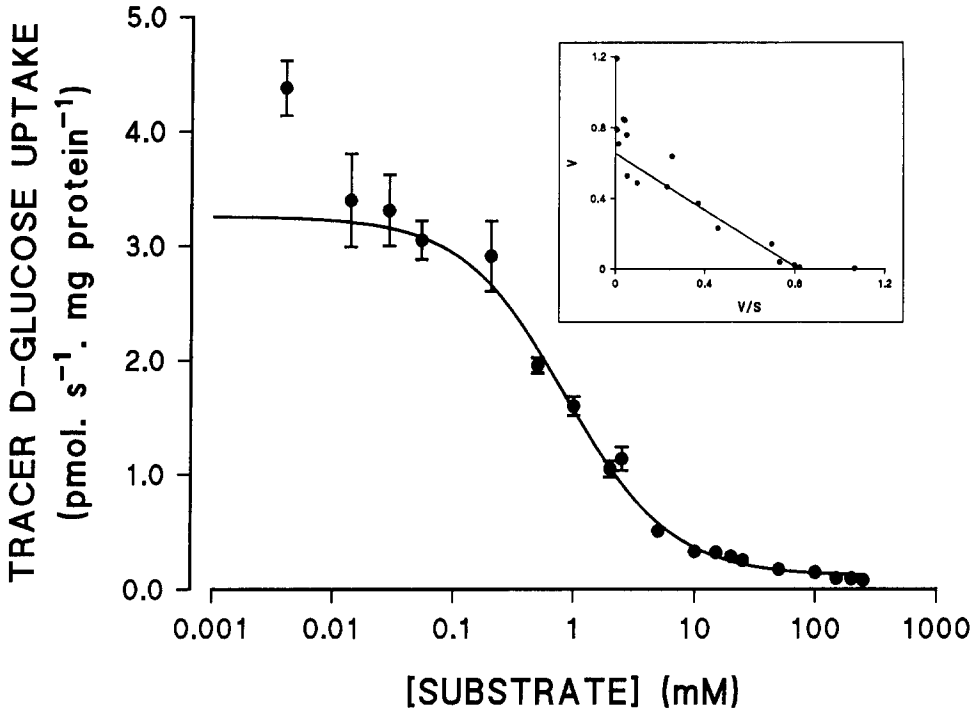

Fig. 4. Determination of kinetic parameters of D-glucose transport in adult guinea-pig BBMV at 35°C. The following experimental conditions were used: The resuspension medium contained 10 mM HMBA buffer (pH 7.4) and 500 mM D-sorbitol. The final concentrations in the incubation media were: 10 mM HMBA buffer (pH 7.4), 100 mM NaCl, 4 μM D-[^{3}H]glucose and increasing amounts of unlabeled D-glucose (0 to 250 mM). Osmolarity of the external media was maintained with D-sorbitol to a fixed total sugar concentration of 300 mM. Non-linear regression analysis gave K_m, V_{max} and K_d values of 0.80 ± 0.10 mM, 655 ± 60 pmol/s per mg protein, and 0.12 ± 0.02 pmol/s per mg protein at 4 μM tracer, respectively. Each data point represents the initial rate of D-glucose uptake $\pm$S.D. of regression. Each experiment was performed in triplicate. The inset shows the Eadie-Hofstee transformation of data. See Results for more details.

TABLE II

Effects of stop solutions on D-glucose uptake measurements in different BBMV preparations

Brush-border membranes were precipitated with either $CaCl_2$ or $MgCl_2$ and vesicles were resuspended in 50 mM Tris-Hepes (TH) buffer (pH 7.5), 0.1 mM $MgSO_4$, 100 mM KCl and 500 mM D-mannitol (a); or 10 mM Hepes-maleic acid-butylamine (HMBA) buffer (pH 7.4), 100 mM KCl and 500 mM D-sorbitol (b). Final concentrations in the incubation media were: (a) 50 mM TH buffer (pH 7.5), 0.1 mM $MgSO_4$, 96 mM NaCl, 4 mM KCl, 500 mM D-mannitol and 4 μM D-[^{3}H]glucose; (b) 10 mM HMBA buffer (pH 7.4), 96 mM NaCl, 4 mM KCl, 500 mM D-sorbitol and 4 μM D-[^{3}H]glucose. Isosmolar stop solutions contained: (a) 50 mM TH buffer, 0.1 mM $MgSO_4$, 100 mM NaCl, 500 mM D-mannitol and 1 mM phlorizin (PZ); (b) 10 mM HMBA buffer, 100 mM NaCl, 500 mM D-sorbitol and 1 mM PZ. The hyperosmolar stop solution contained: 10 mM HMBA buffer, 25 mM $MgSO_4$ and 350 mM KCl as described elsewhere (Ref. 22). Percentages of decrease relative to the TH condition are given in parentheses. [c] Initial rate of D-glucose uptake (pmol/s per mg protein).

Buffer	Stop solution	$MgCl_2$	$CaCl_2$
TH [a]	isosmolar + PZ	5.4 ± 0.2 [c]	4.2 ± 0.1
HMBA [b]	isosmolar + PZ	1.9 ± 0.1 (-65%)	1.6 ± 0.1 (-62%)
HMBA	hyperosmolar	1.5 ± 0.1 (-72%)	1.2 ± 0.1 (-71%)

Purity of D-mannitol and D-sorbitol preparations

D-Glucose concentration was measured in D-sorbitol obtained from 3 different sources (Sigma, Aldrich and Fisher) and found to represent 0.042%, 0.033% and

TABLE III

Effects of D-mannitol, D-sorbitol and buffers on the initial rate of D-glucose uptake

Brush-border membranes were precipitated with $MgCl_2$ and vesicles were resuspended in either Tris-Hepes (TH) or HMBA buffers, as described in the caption to Table II. In both cases, 500 mM of either D-mannitol or D-sorbitol was used to maintain osmolarity. TH: Tris-Hepes buffer, 50 mM (pH 7.5). HMBA: Hepes, maleic acid, n-butylamine, 10 mM (pH 7.4).

Buffer	Sugar (500 mM)	Initial rate of uptake (pmol/s per mg protein)	Δ
TH	mannitol	73.64 ± 2.05	-66% [a]
	sorbitol	25.14 ± 1.77	
HMBA	mannitol	56.96 ± 2.21	-57% [a]
	sorbitol	24.51 ± 0.42	

[a] % of decrease under sorbitol vs. mannitol conditions.

0.049%, respectively, for the three batches tested. When working with 500 mM sorbitol inside the vesicles, as previously reported [22], these percentages would represent about 200 μM D-glucose. On the other hand, D-glucose was not detectable in mannitol by either enzymatic assay or HPLC (data not shown).

By using the glucose oxydase reaction which transforms D-glucose into glucono-δ-lactone and gluconic acid, contamination of the sorbitol solution by D-glucose was reduced to 0.005%, which represents 25 μM D-glucose at 500 mM D-sorbitol. However, since the initial rate of D-glucose uptake was reduced by 37% and 39% at 20 and 35°C, respectively, in the presence of 1 mM D-gluconic acid (data not shown), the end products of the glucose oxydase reaction were removed as described in Materials and Methods. Less than 0.015% residual contaminants were found after ion exchange chromatography.

Effects of purified sorbitol on D-glucose uptake

The initial rates of D-glucose uptake were then measured at both 20 and 35°C in the presence of 500 mM of either D-mannitol or purified D-sorbitol in the incubation medium. D-Glucose uptake inhibition was reduced from 66% (Table III) to an average of 15% after removal of most of the contaminants present in sorbitol (initial rates of D-glucose uptake were 6.6 ± 0.5 vs. 5.5 ± 0.6 pmol/s per mg protein, at 20°C; and, 23.7 ± 0.5 vs. 21.1 ± 0.1, at 35°C, in D-mannitol and purified D-sorbitol media, respectively). This residual inhibition of D-glucose uptake was accounted for by the small amount of D-glucose (25 μM) still present in sorbitol after the purification procedure. A similar reduction (18%) of tracer D-glucose uptake was observed in the course of our kinetic experiments when 25 μM unlabeled D-glucose was added to the incubation medium.

As noted before for V_{max} values (Table I), the initial rate of D-glucose uptake was also increased with temperature; using the same vesicle preparation, a 3.4-fold rise in the initial rate value was recorded at 35°C (6.9 ± 0.2 vs. 23.7 ± 0.5 pmol/s per mg protein).

Discussion

As discussed elsewhere [20,30], the use of both a non-linear regression procedure and a displacement curve of tracer uptake by an unlabelled substrate offers numerous advantages in the analysis of transport data. This procedure has been used recently to show the presence of both a high- and a low-affinity transport system for L-glutamic acid in rabbit jejunal BBMV [35]. By using this approach and a FSRFA [24], we confirmed that guinea-pig small intestine can accumulate sugar before birth [12] and we demonstrated that a single Na^+/D-glucose cotransporter is responsible for this uptake at 20°C in jejunal BBMVs isolated at various stages of development.

Since Brot-Laroche et al. [22] reported that a S-2, high temperature-sensitive system is expressed at 35°C in the adult guinea-pig jejunum, kinetic studies were also performed at this temperature. However, no evidence of a second system for D-glucose uptake was obtained at 35°C; the Eadie-Hofstee plots were linear and the Hill numbers were not statistically different from 1. Another uptake component was also measured at 35°C (Fig. 2 and Table I), although it was undetectable at 20°C (Fig. 1 and Table I). This uptake component cannot be part of a second specific low affinity system since it was shown to be equivalent to simple diffusion of substrate, as evaluated by the uptake of L-glucose, D-glucose in the absence of Na^+ and tracer D-glucose in the presence of Na^+ and saturable concentration of unlabeled substrate (Fig. 3). One can also note that the K_m value was unaffected by temperature although a 4-fold increase of V_{max} was noted at 35°C. A similar increase of the V_{max} value for D-glucose uptake as a function of temperature has also been observed in the adult human jejunum but no evidence of a second system with a low-affinity for D-glucose was obtained [20]. These results contrast with those reported for adult guinea-pig BBMV [22], in which a single system with a K_m of 1.2 mM was noted at 25°C and two systems with K_m values of 0.4 and 24 mM were found at 35°C.

Could our different results be explained by a loss of transport activity during the experimental process? Such a decline in uptake activity has already been reported in both rabbit and rat BBMV [27] and we have observed similar behaviour in unfrozen guinea-pig vesicles. Obviously, uptake measurements need to be performed with stable membrane preparations for accurate determination of the kinetic characteristics of any transport system [30]. Such difficulties were solved in our experiments by freezing BBMV fractions in liquid nitrogen until each assay was conducted, a procedure which prevents any variation in uptake measurements [27]. A similar procedure was also used in experiments of Brot-Laroche et al. [22,23], thus ruling out the possibility that freezing could inactivate the low-affinity system already seen by these authors.

Another important factor for the precise determination of substrate uptake into brush-border membrane vesicles is the efficiency of the stop solution in preventing any efflux of the labelled substrate from the vesicles [30]. Our results indicate that the stop solution was not the major factor responsible for the large decrease in D-glucose uptake recorded under such conditions (Table II) but point to an important role of the HMBA-sorbitol medium in this regard (Table III). More precisely, D-sorbitol can be identified as the unique factor responsible for the large drop in D-glu-

cose uptake (Tables II and III) at any temperature and under different membrane potentials.

At first sight, it is difficult to conceive that D-sorbitol would interfere with D-glucose transport, and D-mannitol, which is structurally similar to sorbitol, would be inert. In fact, these sugars differ only in the position of the OH group on carbon-2. As defined by Crane [35], actively transported sugars possess a common pyranose ring, a methyl or substituted methyl group at carbon-5 of the ring and a hydroxyl group in the glucose configuration at carbon-2. Neither mannitol nor sorbitol meets these structural requirements for specific interaction with the Na^+/D-glucose cotransporter at the brush-border membrane level. However, the observed effect of increasing concentrations of D-sorbitol on the initial rate of D-glucose uptake (apparent K_i value of 64 ± 15 mM) suggests such an interaction.

Looking forward to a rational explanation for this unexpected behaviour, we compared the chemical processes used in the commercial preparation of these sugars. In both cases, electrolytic reduction is used; mannitol is prepared from mannose and sorbitol from glucose. It thus appears likely that mannose and glucose could represent trace contaminants in mannitol and sorbitol solutions, respectively. Since mannose and mannitol are not actively transported sugars [38], no inhibition of D-glucose uptake should be expected. On the other hand, trace amounts of D-glucose would affect labeled D-glucose uptake measurements. In fact, after removal of nearly all D-glucose and the end products of the glucose oxydase reaction in sorbitol, non-significant inhibition of D-glucose uptake was detected. It can thus be concluded that sorbitol per se does not affect D-glucose transport in BBMV but, from a practical point of view, its use as a replacing sugar in such experiments must be avoided. Indeed, loading vesicles with 500 mM D-sorbitol and studying D-glucose uptake in different incubation media containing increasing concentrations of D-glucose and, parallely, decreasing amounts of sorbitol could introduce: (i) trans-inhibitory effects at low concentrations of external D-glucose; (ii) underestimation of the total amount of substrate in the incubation medium; and (iii) progressive 'inhibition' release when reducing external concentrations of D-sorbitol. Such artifacts can explain the very low uptake values recorded in the presence of sorbitol and the misestimation of kinetic parameters. When glucose concentrations were recalculated, assuming both internal and external contamination, a K_m of 0.55 ± 0.1 mM instead of 0.8 mM was obtained.

As pointed out elsewhere [20,30], the misestimation of non specific uptake components can led to an erroneous interpretation of kinetic data. Such problems could be better solved by non linear regression analysis and a displacement curve, as argued previously [20,30] and applied here. Moreover, our study confirms that there is no detectable Na^+-independent pathway for D-glucose uptake in guinea-pig small intestine (Fig. 3) and that passive diffusion fully account for the non-specific component observed at 35°C (Fig. 2). This diffusion pathway appears to be highly sensitive to temperature, since it was undetectable at 20°C and clearly measurable at 35°C, as illustrated in Figs. 1–3 and reported in Table I. Similar behaviour was also observed in a previous study using adult human BBMV [20]. The fact that the two-site equation model can be fitted to data obtained in the presence of sorbitol without any diffusional component, and that the model for two Michaelian components working with a non specific one cannot account for the same data, suggests that the S-2 system identified as such by Brot-Laroche et al. [22] could be part of the non specific uptake component.

Despite all the above arguments, one cannot definitively exclude the possibility that a second Na^+/D-glucose cotransport system exists in the small intestine. However, the technique of tracer uptake measurements reaches a limit where the active transport, non specific component and background values can hardly be distinguished from one another. Other approaches, such as those provided by molecular biology, would certainly help to elucidate this crucial point. The cloning of a rabbit intestinal cotransporter (SGLT1) [39], which corresponds to a high-affinity system, has been successful five years ago. Since this time, however, in situ hybridization and immunocytochemical techniques using both cDNA probes and antibodies against SGLT1 have failed to confirm the presence of both high- and low-affinity pathways in rat [40] and rabbit [41] small intestine.

In conclusion, a single Na^+/D-glucose cotransport system appears to be present throughout life in the guinea-pig jejunum at both temperatures studied. Our experiments also point out that the utilization of D-sorbitol to maintain medium osmolarity must be avoided when studying D-glucose or related sugar transport.

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