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Separation of two distinct Na^+/D-glucose cotransport systems in the human fetal jejunum by means of their differential specificity for 3-*O*-methylglucose

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Based on kinetic arguments, we have recently proposed the existence of two distinct Na^+/D-glucose cotransporters in brush-border membrane vesicles isolated from the human fetal jejunum (Biochim. Biophys. Acta 938 (1988) 181–188). In order to further test this hypothesis, inhibition studies of the zero-trans influx of substrate have been performed under Na^+-gradient and voltage-clamped conditions. Initial rates of D-glucose uptake were totally abolished by D-glucose, D-galactose, α-methylglucose and phlorizin while 3-*O*-methylglucose and phloretin induced only a 65% inhibition even at the highest concentrations used. The residual activity of D-glucose uptake is thus compatible with substrate flux through a low-affinity transport system which is insensitive to phloretin and does not accept 3-*O*-methylglucose as substrate. This substrate specificity has been used to separate kinetically the two putative pathways for glucose transport. The data obtained are compatible with the existence of the following two systems: (i) a low-affinity, high-capacity system with a K_m of 4.7 mM and a V_{max} of 22 nmol/min per mg of protein, and; (ii) a high-affinity, low-capacity system with a K_m of 0.57 mM and a V_{max} of 10.7 nmol/min per mg of protein. These data thus demonstrate clearly the existence of two distinct Na^+-dependent D-glucose carriers in the human jejunum during the early gestation period since these systems can be differentiated not only by their kinetic properties but also by their differences in both substrate and inhibitor specificities.

Introduction

In a previous study [1], we have provided kinetic evidence as to the existence of two distinct Na^+-dependent D-glucose carriers in the jejunum of the 17–20-week-old human fetus. Our data supported the presence of both: (i) a high-affinity, low-capacity system with a K_m of 0.37 mM, a V_{max} of 8.3 nmol/min per mg protein and a minimal stoichiometry of 2 Na^+ for 1 glucose, and, (ii) a low-affinity, high-capacity system with a K_m of 4.2 mM, a V_{max} of 30.9 nmol/min per mg protein and a stoichiometry of 1 Na^+ for 1 glucose. These results are in agreement with kinetic studies performed in bovine [2], rabbit [3] guinea-pig [4] and adult human [5] small intestinal brush-border membrane vesicles as well as in hamster everted gut sacs [6]. All together, these results have led to the general opinion that there is an heterogeneity of glucose carriers in the small intestine, as it is the case for the rabbit kidney where two distinct Na^+/D-glucose cotransport systems have been identified, based on their kinetic as well as their anatomical separation [7]. However, no such clear demonstration has been reported for the Na^+-dependent D-glucose carrier(s) in the small intestine, the proof being based solely on kinetic analysis of curvilinear Eadie-Hofstee plots. Only in the case of the hamster small intestine, additional evidence for two systems has been provided by differential substrate specificity for 6-deoxyglucose and D-glucose [8]. Nevertheless, these arguments by themselves do not definitively prove the existence of multiple pathways for glucose transport in the small intestine.

Indeed, even though kinetic studies support an heterogeneity of Na^+-dependent D-glucose carriers in the

Abbreviations: Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone.

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small intestine, one has to be careful in interpreting kinetic data as such. In a recent review [9], Sanders has pointed out some possible misinterpretation of kinetic data which can be obtained depending of the experimental conditions used. Sanders clearly shows that a curvilinear Eadie-Hofstee plot can be obtained with a random cotransport model when the cosubstrate, that is Na^+, is not saturating. As such, one has to be very careful in interpreting kinetic data and analyzing curvilinear plots using high performance curve-fitting programs which allow the mathematical separation of eventually more than two distinct systems, which maybe without any physiological meanings. All together, these arguments point to further characterization of our systems in terms of substrate and inhibitor specificity in order to definitively prove their common existence.

Furthermore, a complete study of substrate specificity could be helpful in understanding the symptoms of children with glucose-galactose malabsorption syndrome. Indeed, in order to explain the course of this disease, in which affected children cannot tolerate either sugars at birth but become able to ingest glucose after a few years of age, Semenza and Corcelli [10] have proposed the following hypothesis: at birth, the human gut has a single glucose-galactose transporter, and this transporter is lacking in glucose-galactose malabsorption syndrome while another carrier, specific for D-glucose, develops after a few years of age. Even though our kinetic data concerning the presence of two distinct carriers in the human fetus argue against this developmental pattern, the conclusions drawn in the absence of substrate specificity study could lead to erroneous interpretation.

The aim of the present study was then to clearly define the functional properties of the Na^+/D-glucose cotransport system(s) in the brush-border membrane from human fetal jejunum in order (i) to definitively demonstrate their common existence, and (ii) to get a better understanding of the functional development of the human small intestine.

Materials and Methods

Chemicals

Labeled compounds, namely D-[U-^{14}C]glucose (250 mCi/mmol) and D-[1(n)-^{3}H] mannitol (19.1 mCi/mmol) were purchased from New England Nuclear Corporation; D-[1-^{14}C]mannitol (59 mCi/mmol), 3-*O*-methyl-D-[1-^{3}H]glucose (8.5 Ci/mmol), and ^{22}Na, carrier free (100–1000 mCi/mg) were purchased from Amersham Canada Ltd. Valinomycin was obtained from Sigma Chemical Co.; FCCP, phlorizin and phloretin were purchased from Aldrich Chemical. All salts and chemicals used for buffer preparation were of the highest purity available.

Preparation of brush-border membrane vesicles

Fresh, 17–20-week-old normal human fetal small intestines were kindly provided by Dr. Jean Michaud from Ste-Justine Hospital where social abortions have been performed. The jejunum were collected and prepared as previously described [11]. Brush-border membranes were purified by the $CaCl_2$ precipitation method of Schmitz et al. [12] and vesicles were prepared as described previously [1]. The purity of the brush-border membrane fractions was routinely tested by determination of both apical (sucrase) and basolateral (Na^+/K^+-ATPase) membrane markers activities. An enrichment factor of 17–22-fold over the homogenate was obtained and contamination by basolateral membranes was always less than 5%.

Transport studies

The uptake of D-[^{14}C]glucose, 3-*O*-methyl-D-[^{3}H]glucose and ^{22}Na was performed using a manual rapid filtration technique as described previously [1], or a newly designed fast sampling, rapid filtration apparatus (manuscript in preparation). Briefly, this apparatus allows multiple sampling from the same incubation mixture at very short period of time (1 s); the filtration and washing steps are performed automatically within 12 s. Thus, the determination of initial rates can be made by linear regression analysis over multiple points obtained in the linear portion of the uptake time curves. Using the manual rapid filtration procedure, initial rates of D-glucose uptake were determined at 6 s under Na^+-gradient conditions with membrane potential clamped to zero by K^+-valinomycin (5 μM) and H^+-FCCP (10 μM) as described previously [1]. We have already shown that the time course of D-glucose uptake was linear up to at least 30 s [11]. All data were corrected for the dead space as measured with radiolabelled mannitol added to the ice-cold stop solution. For kinetic experiments, data were also corrected for the non-specific component as determined after inhibition of the Na^+-dependent D-glucose uptake by 1 mM phlorizin. The osmolarity of the incubation media was maintained with D-mannitol. The exact composition of the resuspension and incubation media are given in the legends of the figures. Results are expressed as nmol solute uptake per mg protein. Curvilinear Eadie-Hofstee plot were analyzed by a non-linear least-squares curve-fitting iterative procedure based on Feldman's parameter fitting [13] using an Apple II desk computer.

Results

Inhibition studies

Initial rates of D-[^{14}C]glucose transport have been determined as a function of increasing concentrations (from 5 to 100 mM) of various sugars in the incubation

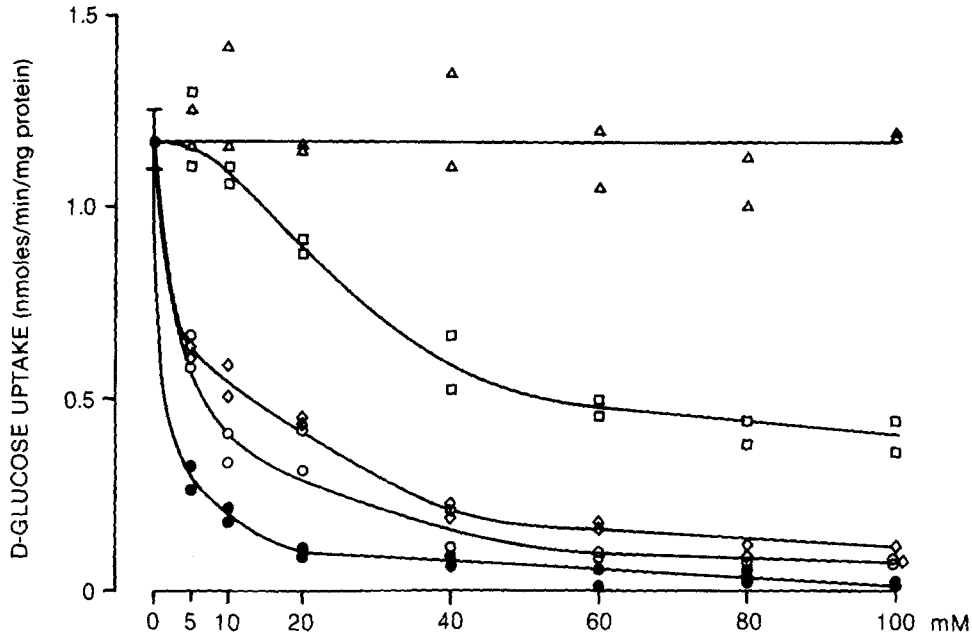

Fig. 1. Inhibition of the initial rate of D-glucose transport at 100 μM by increasing concentration of D-glucose (●), D-galactose (○), α-methyl-D-glucose (◇), 2-deoxyglucose (△) and 3-*O*-methylglucose (□). Vesicles were resuspended in 50 mM Tris-Hepes buffer (pH 7.5), 0.1 mM $MgSO_4$, 250 mM CholCl, 100 mM KCl, 125 mM mannitol, 5 μM valinomycin and 10 μM FCCP. The final concentrations in the incubation media were: 50 mM Tris-Hepes buffer, 0.1 mM $MgSO_4$, 200 mM NaCl, 50 mM CholCl, 100 mM KCl, 100 μM D-[^{14}C]glucose and various concentrations of inhibitors. The osmolarity was maintained by varying mannitol. Each data point represents a single determination from a pool of seven different tissues.

medium. Fig. 1 illustrates the effects of D-glucose, D-galactose, α-methyl-D-glucose, 3-*O*-methylglucose and 2-deoxyglucose as transport inhibitors. D-glucose, D-galactose and α-methyl-D-glucose completely abolish D-[^{14}C]glucose transport while 2-deoxyglucose was completely ineffective. However, 3-*O*-methylglucose induces

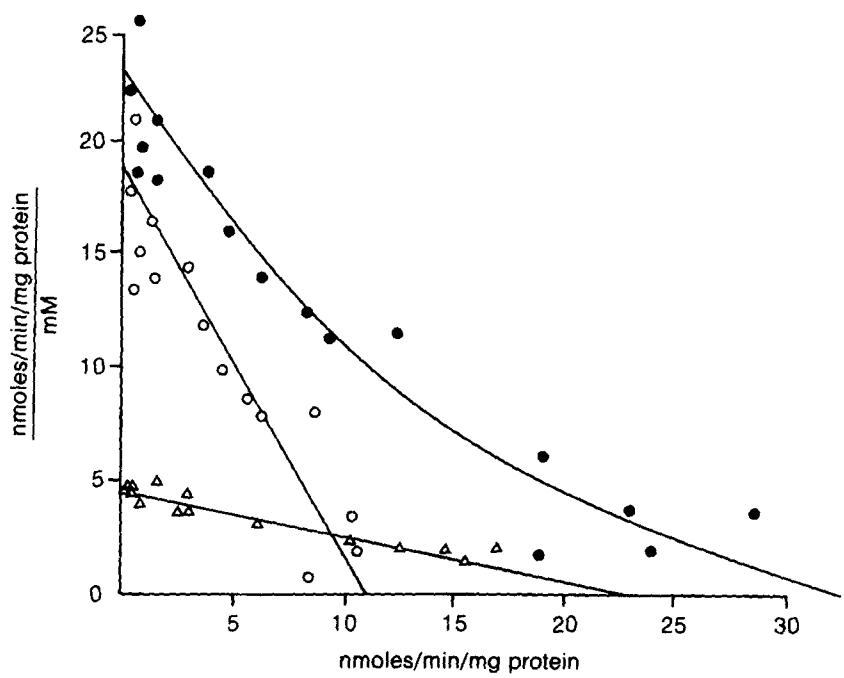

Fig. 2. (Modified) Eadie-Hofstee plot of Na^+/D-glucose cotransport in human fetal jejunum. Resuspension medium: 50 mM Tris-Hepes buffer (pH 7.5), 0.1 mM $MgSO_4$, 100 mM KCl, 400 mM CholCl, 5 μM valinomycin and 10 μM FCCP. Resuspension media (final concentrations): 50 mM Tris-Hepes buffer, 0.1 mM $MgSO_4$, 100 mM KCl, 200 mM NaCl, 20 μM up to 12 mM of both D-[^{14}C]glucose and unlabelled D-glucose, and either 200 mM CholCl (●) or 150 mM CholCl and 100 mM 3-*O*-methylglucose (△). Calculated values (○) obtained by substraction of the uptake equation in presence of 100 mM 3-*O*-methylglucose (△) from the total uptake curve (●). Each data point represents a single determination with a given concentration of D-glucose.

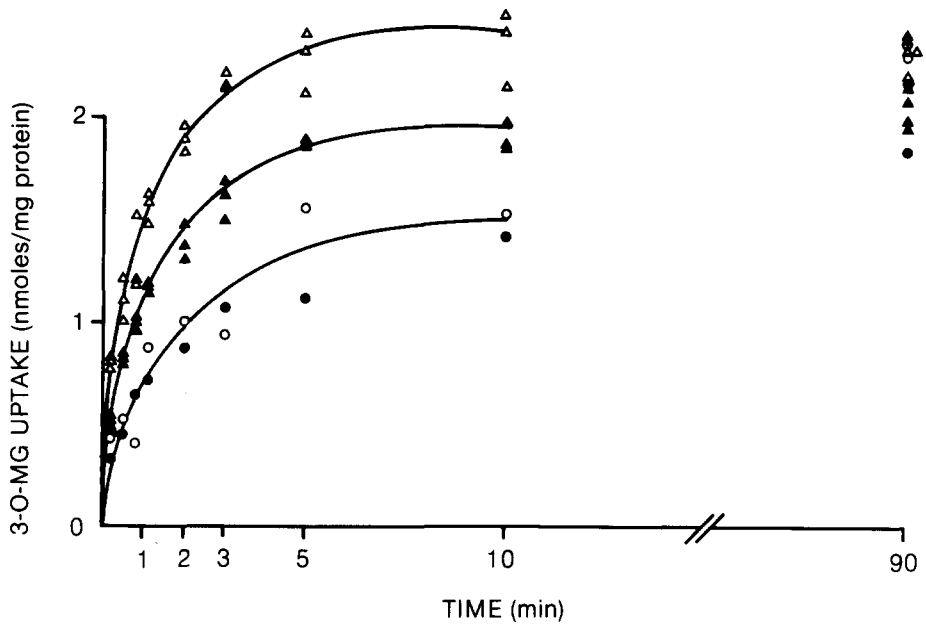

Fig. 3. Accelerated exchange of 3-*O*-methyl-D-glucose in human fetal jejunum. Vesicles were resuspended in 50 mM Tris-Hepes buffer (pH 7.5), 0.1 mM $MgSO_4$, 100 mM KCl, 200 mM CholCl, 5 μM valinomycin, 10 μM FCCP and either 20 mM Mannitol (▲, ●) or 20 mM 3-*O*-methylglucose (△, ○). The final concentrations in the incubation media were: 50 mM Tris-Hepes buffer, 0.1 mM $MgSO_4$, 100 mM KCl, 192 mM NaCl, 8 mM CholCl, 20 μM 3-*O*-methyl[^{3}H]glucose, 800 μM unlabelled 3-*O*-methylglucose, 19.2 mM mannitol, without (▲, △) or with 1 mM phlorizin (●, ○). Each data point represents a single determination.

only a partial inhibition of D-glucose transport with the residual activity at 100 mM corresponding to 35% of the total transport activity.

TABLE I

Functional characteristics of Na^+/D-glucose cotransport systems in the human fetal jejunum

Results are expressed as means ± S.E. HAS, high-affinity system; LAS, low-affinity system.

	HAS	LAS
K_m for D-glucose (mM)	0.57 + 0.06 [a]	4.7 + 0.5
	0.45 + 0.13 [b]	4.5 + 1.5
	(0.37 + 0.06) [c]	(4.2 + 1.0)
V_{max} (nmol/min per mg protein)	10.7 + 0.8 [a]	22.0 + 2.0
	7.5 + 2.9 [b]	33.9 + 5.3
	(8.3 + 2.3) [c]	(30.9 + 1.9)
Stoichiometry Na^+/glucose	2:1	1:1
Inhibitors	phlorizin	phlorizin
	phloretin	-
Substrates	D-glucose	D-glucose
	D-galactose	D-galactose
	α-methylglucose	α-methylglucose
	3-*O*-methylglucose	-

[a] Values obtained after inhibition of the HAS by 100 mM 3-*O*-methylglucose.
[b] Values determined by direct analysis of the curvilinear Eadie-Hofstee plot (this study).
[c] Values determined in a previous study [1].

Kinetic studies

In our previous studies [1], we have determined the kinetic parameters of the two putative Na^+/D-glucose cotransport systems present in the human fetal jejunum. A similar study has been performed in the presence and absence of 100 mM 3-*O*-methylglucose in the incubation medium. Fig. 2 illustrates the modified Eadie-Hofstee representation of Na^+-dependent D-glucose uptake as a function of increasing concentration of D-glucose in the incubation medium, ranging from 20 μM up to 12 mM. In the absence of 3-*O*-methylglucose, the plot is curvilinear and can be revolved in both a high-affinity, low-capacity system with a K_m of 0.45 mM and a V_{max} of 7.5 nmol/min per mg protein, and a low-affinity, high-capacity system with a K_m of 4.5 mM and a V_{max} of 33.9 nmol/min per mg protein (Fig. 2 and Table I). These values are closed to the one previously reported [1]. When these determinations were repeated in the presence of saturating concentration of 3-*O*-methylglucose in the incubation medium (opened triangles), the plot becomes linear with a K_m of 4.7 mM and a V_{max} of 22 nmol/min per mg protein which are close to the kinetic parameters determined from the total curve for the low-affinity system (Table I). When this single component is subtracted from the total curve, another single component can be isolated (opened circles) with 0.57 mM and 10.7 nmol/min per mg protein for K_m and V_{max}, respectively.

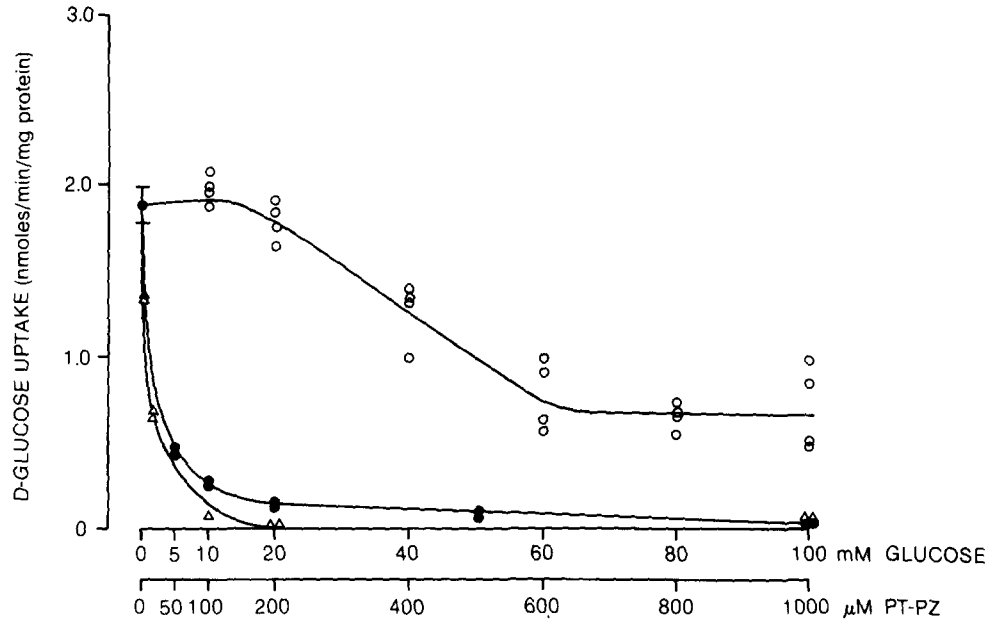

Fig. 4. Inhibition of the initial rate of D-glucose uptake at 100 μM by increasing concentrations of phlorizin (△), phloretin (○) (from 1 μM up to 1 mM), and D-glucose (●) (5–100 mM). Both resuspension and incubation media were as described in Fig. 1. Each data point represents a single determination.

Transport of 3-O-methyl-D-glucose

In order to determine whether 3-*O*-methylglucose is a transported substrate or inhibits D-glucose uptake by binding to the transporter in the absence of translocation, the time course of 3-*O*-methyl[^{3}H]glucose uptake has been determined under both zero-trans and accelerated exchange conditions. These results are illustrated in Fig. 3. In zero-trans condition (filled triangles), 3-*O*-methylglucose accumulates within the vesicles over a 10 min period. This uptake of 3-*O*-methylglucose is increased by the presence of 20 mM 3-*O*-methylglucose inside the vesicles (opened triangles) and in both zero-trans (filled circles) and accelerated exchange (opened circles) conditions, this uptake appears sensitive to

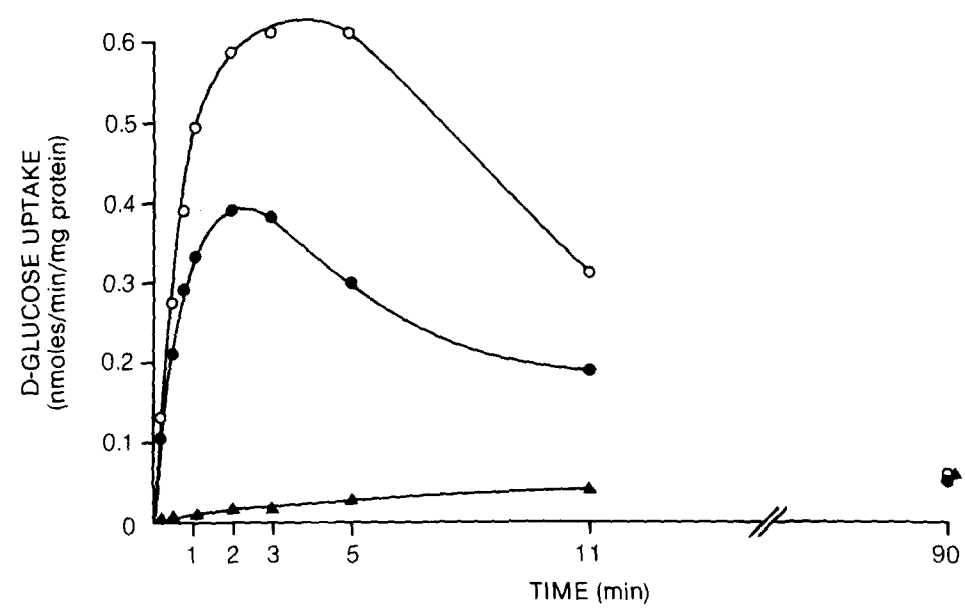

Fig. 5. Time course of D-glucose uptake into brush-border membrane vesicles isolated from human fetal jejunum. Vesicles were resuspended in 50 mM Tris-Hepes buffer (pH 7.5), 0.1 mM $MgSO_4$ and 250 mM KCl. Incubation media contained 50 μM D-[^{14}C]glucose, 50 mM Tris-Hepes buffer, 0.1 mM $MgSO_4$ and either 250 mM KCl (▲), or 200 mM NaCl and 50 mM KCl without (○) or with 200 μM phlorizin (▲) or 200 μM phloretin (●). Points shown are individual data points from the same preparation of vesicles and are representative of the three experiments performed under identical conditions.

phlorizin. These data indicate a transport of 3-*O*-methylglucose rather than a binding to the cotransporter without translocation of the inhibitor.

Effects of phlorizin and phloretin

In order to evaluate the sensitivity of D-glucose transport to both phlorizin and phloretin, the initial rates of D-[^{14}C]glucose transport were determined as a function of increasing inhibitors (from 1 μM up to 1 mM) and unlabeled D-glucose (5–100 mM) concentrations in the incubation medium. As illustrated in Fig. 4, D-glucose transport is completely inhibited by both D-glucose (filled circles) and phlorizin (triangles). However, phloretin (opened circles) induces only a partial inhibition of D-glucose transport; here again, the residual activity at 1 mM represents 35% of the total transport, as observed previously for the inhibition of D-glucose transport by 3-*O*-methylglucose (Fig. 1).

The time course of Na^+/D-glucose uptake has then been evaluated in the human fetal jejunum in the presence of 200 μM phlorizin or phloretin. As illustrated in Fig. 5, Na^+-dependent D-glucose uptake (opened circles) is completely inhibited by phlorizin (filled triangles), the level obtained being the one observed in the complete absence of Na^+ (filled triangles). By contrast, phloretin (filled circles) at the same concentration induces only a partial inhibition of D-glucose accumulation within the vesicles.

The effect of 1 mM phloretin on the initial rate of 3-*O*-methylglucose uptake under accelerated exchange condition has also been studied. As showned in Fig. 6, the uptake of 3-*O*-methylglucose (opened squares) is linear ($r = 0.993$) over a 5-s period and is inhibited to the same extent (58% inhibition) by either phlorizin (closed circles) or phloretin (opened circles). The uptake of 3-*O*-methylglucose is also linear ($r = 0.923$) in the presence of both inhibitors.

Effects of both substrate and inhibitors on Na^+ permeability

The Na^+ permeability of brush-border membrane vesicles, incubated in the presence of 100 mM D-glucose, 1 mM phlorizin or 1 mM phloretin, was evaluated directly by measuring ^{22}Na uptake under conditions identical to those described in Fig. 1. Initial rates of ^{22}Na uptake were determined by linear regression analysis on the linear portion (up to 30 s) of the uptake-time

TABLE II

Initial rates of ^{22}Na uptake under various conditions

Results are expressed as nmoles of ^{22}Na transported per min per mg protein. Values are means ± S.E. for three different preparations of vesicles. Initial rates were determined by linear regression analysis over the linear portion of the uptake-time curves. Resuspension and incubation media were as described in the legend of Fig. 1, except for the replacement of radiolabelled D-glucose by ^{22}Na.

Addition	^{22}Na uptake
D-Glucose (50 μM)	228.0 ± 40.8
D-Glucose (100 mM)	205.8 + 46.2
Phlorizin (1 mM)	216.0 ± 52.8
Phloretin (1 mM)	236.4 ± 51.0

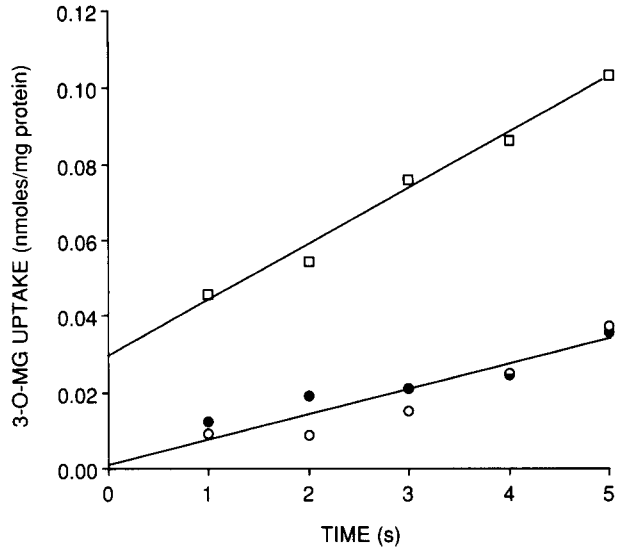

Fig. 6. Effects of phlorizin and phloretin on the initial rate of 3-*O*-methylglucose uptake under accelerated exchange condition. Resuspension medium was as described in Fig. 3, with 20 mM 3-*O*-methylglucose. Incubation media were as described in the legend of Fig. 3, without (□) or with 1 mM of either phlorizin (●) or phloretin (○). These uptake-time curves were obtained using a fast sampling, rapid filtration apparatus, as described under Materials and Methods. Linear regression was performed as described in the text and gave y intercepts, slopes and coefficients of correlation of $2.9 \cdot 10^{-2}$, $1.5 \cdot 10^{-2}$, 0.993 (□), and $1.9 \cdot 10^{-3}$, $6.3 \cdot 10^{-3}$, 0.923 (●, ○) respectively.

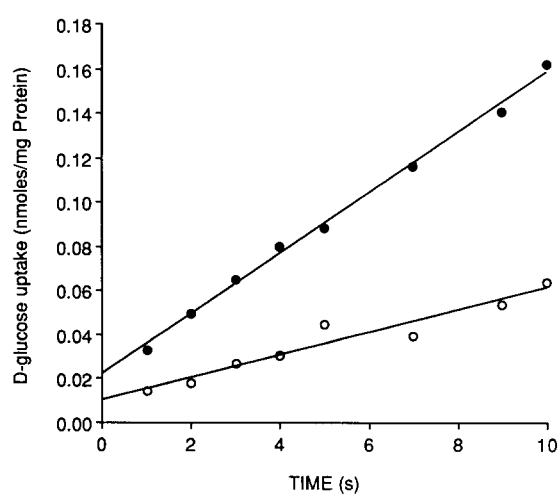

Fig. 7. Effect of phloretin on the initial rate of D-glucose uptake. The initial rate of D-glucose uptake was determined in the absence (●) or in the presence (○) of 1 mM phloretin over a 10-s period, using a fast sampling, rapid filtration apparatus. Both resuspension and incubation media were as described in Fig. 1. Linear regression analysis gave y intercepts, slopes and coefficients of correlation of $2.2 \cdot 10^{-2}$, $1.4 \cdot 10^{-2}$, 0.998 for control (●) and $1.0 \cdot 10^{-2}$, $5.1 \cdot 10^{-3}$, 0.964 for phloretin (○) conditions, respectively.

curves. These determinations were done in duplicate on three different preparations of brush-border membrane vesicles. As shown in Table II, there is no significant differences in initial rates of ^{22}Na uptake under any of the conditions tested.

In order to further demonstrate that a faster dissipation of the Na^+-gradient cannot be responsible for the partial inhibition observed in the presence of 1 mM phloretin, the initial rate of D-glucose uptake was determined over a 10-s period. As shown in Fig. 7, D-glucose uptake is linear during this time interval in both control (filled circles; $r = 0.998$) and phloretin conditions (opened circles; $r = 0.964$). Furthermore, the initial rate of D-glucose uptake in the presence of 1 mM phloretin (0.304 ± 0.034 nmol/min per mg protein) represents only 37% of the total uptake (0.822 ± 0.023 nmol/min per mg protein), as reported previously in Fig. 4.

Discussion

In this paper, we have characterized two distinct Na^+/D-glucose cotransport systems present in the brush-border membrane from the human fetal jejunum. These systems were already identified in our previous study [1] by differences in their kinetic parameters as well as in their stoichiometries for Na^+. The present data clearly demonstrate their common existence (i) by defining their specificities for both different substrates and inhibitors, and (ii) by using the inhibition of D-glucose transport by 3-*O*-methylglucose to separate these two systems in order to reevaluate their kinetic properties in an independent way.

The inhibition studies of initial rate of D-glucose transport by increasing concentrations of different sugars show complete inhibition by either glucose, galactose and α-methyl-D-glucose but with different ID_{50}. On a millimolar basis, glucose is the most potent and α-methyl-D-glucose the least. 3-*O*-Methylglucose, for his part, induces only a partial inhibition of the initial rate of D-glucose transport, with a residual activity at 100 mM 3-*O*-methylglucose representing 35% of the total transport activity. Taking into account the kinetic parameters previously determined for the two transport systems in the human fetal jejunum [1], we can evaluate that the low-affinity system is responsible for about 29% of the total transport of D-glucose at 100 μM substrate. Then, this residual activity appears compatible with the activity of a single system, that is the low-affinity one, thus suggesting that the high-affinity system would be inhibited by 3-*O*-methylglucose. This apparent specificity for 3-*O*-methylglucose has been already suggested by Honegger and Semenza [6] in the everted sacs from hamster small intestine. These authors have found 2 different Na^+/D-glucose cotransport systems along the length of the small intestine, carrier 1 interacting with glucose, galactose, 6-deoxyglucose and 3-*O*-methylglucose although carrier 2 being specific for glucose and galactose only. This conclusion was based on Eadie-Hofstee plots which were curvilinear for both glucose and galactose and linear for 3-*O*-methylglucose. Similar conclusion can be drawn from our experiments using jejunal brush-border membrane vesicles from human fetus.

This apparent specificity of the high-affinity system for 3-*O*-methylglucose has then been used in an effort to separate the two carriers and to determine their kinetic properties in an independent way. As shown in Fig. 2, the Eadie-Hofstee plot is clearly curvilinear when the two systems are working together, thus confirming our previous results [1]. In this peculiar experiment, the kinetic parameters determined according to Feldman's parameters fitting are similar to the one previously found [1] (Table I). However, when 100 mM 3-*O*-methylglucose is present in the incubation medium, the plot is linear and the kinetic parameters determined by linear regression analysis are very close to those determined for the low-affinity system by mathematical analysis of the complete curve (Table I). By subtraction of this single component from the total curve determined in the absence of 3-*O*-methylglucose, an other single component with a K_m of 0.57 mM and a V_{max} of 10.7 nmol/min per mg of protein is obtained, thus corresponding to the high-affinity system. These data clearly demonstrate that 3-*O*-methylglucose is taken up by the high-affinity system only, the single system that remains active after inhibition by 100 μM 3-*O*-methylglucose being the low-affinity one. Therefore, the inhibition of D-glucose transport by 3-*O*-methylglucose can be used to separate the high-affinity and the low-affinity systems present in the human fetal jejunum.

3-*O*-Methylglucose could inhibit D-glucose transport by the high-affinity system by acting either as a translocated or non-translocated substrate for the sugar binding site. These possibilities have been tested by comparing the time course of 3-*O*-methyl[^{3}H]glucose uptake in zero-trans and in accelerated exchange conditions. The stimulation of 3-*O*-methylglucose uptake by the presence of 20 mM 3-*O*-methylglucose inside the vesicles clearly demonstrate that 3-*O*-methylglucose is a translocated substrate rather than a ligand which inhibits D-glucose uptake by simply binding to the carrier. Moreover, the fact that phlorizin inhibits 3-*O*-methylglucose uptake to the same extent in both accelerated exchange and zero-trans conditions strengthens this conclusion. However, there is not transient intravesicular accumulation of 3-*O*-methylglucose in both conditions, which can suggest a low-affinity constant or a slow turn-over rate for the substrate-carrier complex [14]. Nevertheless, we have demonstrated that: (i) D-glucose uptake is partially inhibited by 100 mM 3-*O*-methylglucose (Fig. 1); (ii) the single system that re-

mains active after inhibition by 3-*O*-methylglucose is the low-affinity one (Fig. 2); and, (iii) 3-*O*-methylglucose is a transported substrate (Fig. 3) inhibited by both phlorizin and phloretin (Fig. 6). All together, these results clearly lead to the conclusion that the high-affinity system can accept D-glucose, D-galactose, α-methyl-D-glucose and 3-*O*-methylglucose as substrates while the low-affinity system do not accept 3-*O*-methylglucose.

Phlorizin is well established as a competitive inhibitor of the brush-border membrane Na^+-dependent D-glucose cotransporter [15] while phloretin is usually considered as a specific inhibitor of the Na^+-independent D-glucose transport present in the basolateral membrane [16]. However, the inhibition of Na^+/D-glucose cotransport by phloretin, even though not widely recognized, has already been reported in hamster [17,18] and rabbit [19] small intestine as well as in both Caco-2 cells and human fetal colon [20]. Our results show that phloretin can inhibit the Na^+-dependent D-glucose transport in the human fetal small intestine since 200 μM phloretin caused a 37% decrease in the Na^+-driven intravesicular accumulation of D-glucose and since increasing concentrations of phloretin inhibited the initial rate of D-glucose transport up to 65%. The inhibition of initial rate of D-glucose transport by phloretin reach a plateau at 600 μM, a greater amount of phloretin does not induce further inhibition. This behavior strongly suggests that the residual activity recorded after phloretin inhibition (similar to the one observed after inhibition by saturating concentration of 3-*O*-methylglucose) would be attributable to the low-affinity system which appears insensitive to phloretin. Moreover, the assignment of phloretin sensitivity to the high-affinity system is supported by the fact that 3-*O*-methylglucose uptake, which is specific to the high-affinity system (Fig. 2) is inhibited to the same extent by both phlorizin and phloretin (Fig. 6) which are, respectively, full and partial inhibitors of D-glucose uptake (Fig. 4) in the adult human small intestine.

The interpretation of such partial inhibition, especially with phloretin which is a lipophilic substance, rely on the demonstration that the Na^+ gradient is not disturbed by any of the experimental conditions used. In fact, high concentration of phloretin, phlorizin or D-glucose do not affect the Na^+ permeability of the brush-border membranes (Table II), thus ruling out a partial inhibition secondary to a faster dissipation of the Na^+ gradient. Moreover, the inhibition of D-glucose transport by phloretin is observed on the Na^+-dependent fraction of D-glucose transport and cannot be attributed to contamination of the brush-border membrane vesicles preparation by basolateral membranes. In fact, we have previously shown [11] by determination of enzymatic membrane marker activities that the contamination by basolateral membranes is always less than 5%, which is confirmed by the lack of inhibition of the initial rate of D-glucose uptake by 2-deoxyglucose (Fig. 1).

Finally, the fact that D-glucose uptake was linear for at least 10 s in both control and phloretin conditions (Fig. 7), represents by itself a sufficient warranty for the existence of a prevailing steady-state and for the validity of the results obtained at 6 s. Thus, these data definitively rules out the possibility that a faster dissipation of the Na^+ gradient could be responsible for the partial inhibition of D-glucose uptake observed in the presence of 1 mM phloretin. So, even though the molecular mechanism for phloretin inhibition is not clearly understood [17,18], it seems that inhibition by phloretin of apical Na^+-dependent D-glucose uptake may represent a more common phenomena than generally assumed and thus could be used as another criterion to discriminate between the two transport systems present in the membrane.

In guinea-pig small intestine, the presence of two distinct sodium-activated D-glucose transport systems has been recently documented [4]. This study demonstrates that the low-affinity system (S-2) is the most sensitive one to modulation by the physiological state and supports the suggestion that the low-affinity system would be adaptive, whereas the high-affinity system would be constitutive. This possibility can also be considered for the human small intestine, at least during the fetal life. However, the functional significance of having two transporters at this early stage of development is not clear. Do both systems remain active in the adult small intestine or does one of them represent a transitory form, specific to the fetal development are questions which cannot be answered in the absence of identification and characterization of the Na^+/D-glucose cotransport system(s) present in the normal adult human tissue. However, the suggestion of Semenza and Corcelli [10] as to the presence of a single glucose-galactose carrier in the human gut at birth and the post-natal development of a D-glucose-specific carrier cannot be totally excluded. In fact, our results clearly demonstrate the existence of two distinct carriers in the human fetal small intestine but both of them are able to accept D-galactose as substrate. We don't know yet if these two carriers are present in the human gut at birth but if so, it would mean that both of them are affected in the glucose-galactose malabsorption syndrome. If such is the case, it could mean that these two carriers are the products of the same gene and that a deletion on this gene will affect both of them. It is presently premature to put forward any conclusion regarding this point.

Nevertheless, our study clearly demonstrates the existence of two distinct Na^+-dependent D-glucose carriers in the developing human small intestine. This conclusion is based on differences in kinetic properties, stoichiometries for Na^+, specificities for substrates and

sensitivities to inhibitors of the high- and low-affinity Na^+-dependent D-glucose carriers, as summarized in Table I. Furthermore, we have been able to separate these systems by using specific inhibition of the high-affinity system by 3-*O*-methylglucose; this approach allowed the determination of kinetic constants which were very close to the one determined by analysis of the complete curve. These results, all together, strongly substantiate our conclusion as to the existence of a heterogeneity of D-glucose carriers in the human fetal jejunum.

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