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Common Characteristics for Na^+-Dependent Sugar Transport in Caco-2 Cells and Human Fetal Colon

Anne Blais, Pierre Bissonnette, and Alfred Berteloot

Membrane Transport Research Group, Department of Physiology, Faculty of Medicine, University of Montreal, Montreal, Quebec H3C 3T8, Canada

Summary. The recent demonstration that the human colon adenocarcinoma cell line Caco-2 was susceptible to spontaneous enterocytic differentiation led us to consider the question as to whether Caco-2 cells would exhibit sodium-coupled transport of sugars. This problem was investigated using isotopic tracer flux measurements of the nonmetabolizable sugar analog α-methylglucoside (AMG). AMG accumulation in confluent monolayers was inhibited to the same extent by sodium replacement, 200 μM phlorizin, 1 mM phloretin, and 25 mM D-glucose, but was not inhibited further in the presence of both phlorizin and phloretin. Kinetic studies were compatible with the presence of both a simple diffusive process and a single, Na^+-dependent, phlorizin- and phloretin-sensitive AMG transport system. These results also ruled out any interaction between AMG and a Na^+-independent, phloretin-sensitive, facilitated diffusion pathway. The brush-border membrane localization of the Na^+-dependent system was inferred from the observations that its functional differentiation was synchronous with the development of brush-border membrane enzyme activities and that phlorizin and phloretin addition 1 hr after initiating sugar transport produced immediate inhibition of AMG uptake as compared to ouabain. Finally, it was shown that brush-border membrane vesicles isolated from the human fetal colonic mucosa do possess a Na^+-dependent transport pathway(s) for D-glucose which was inhibited by AMG and both phlorizin and phloretin. Caco-2 cells thus appear as a valuable cell culture model to study the mechanisms involved in the differentiation and regulation of intestinal transport functions.

Key Words sugar transport · characterization · cell culture (Caco-2) · fetal colon (human) · differentiation · functional development

Introduction

The demonstration that the human colon adenocarcinoma cell line Caco-2 [10] is susceptible to spontaneous enterocytic differentiation [31] has recently designated this cell line as the most relevant in vitro model for studies related to both differentiation and regulation of intestinal absorbo-digestive functions [36]. Confluent monolayers of Caco-2 cells were first reported to exhibit morphological, immunocytochemical and biochemical differentiation patterns characteristic of mature enterocytes [31]. Dome formation [15, 31, 35] and electrical parameter measurements [15] were also compatible with the uniform presence of tight junctions and appreciable cell polarization. However, the question as to whether Caco-2 cells would exhibit Na^+-coupled transport systems with characteristics similar to those found in intestinal brush-border membranes has not been clearly answered yet. Electrical parameter measurements have shown a small Na^+-coupled alanine flux but this system was not investigated further [15]. Na^+-dependent phosphate transport has also been reported to be present in the apical membrane of Caco-2 cells [27]. However, the existence of a Na^+-coupled sugar transport system seems to be denied by the absence of effects on membrane conductance or depolarization following mucosal addition of glucose or phlorizin [15]. This result is surprising in view of the high rate of glucose utilization in these cells [37].

In an effort to better characterize sugar transport functions in Caco-2 cells, we have undertaken systematic studies with nonmetabolizable sugar analogs using isotopic tracer flux measurements as an alternative approach to previous electrophysiological studies [15]. In the present paper, we report some characteristics of α-methylglucoside (AMG) transport in these cells. Our results clearly demonstrate that Caco-2 cells do possess a Na^+-dependent, phlorizin-sensitive pathway for sugar transport. However, this pathway is also sensitive to phloretin. The nature of this Na^+-dependent sugar transport system was thus investigated and we demonstrate shared properties with a glucose transport system normally found in brush-border membranes from 16- to 22-week-old human fetal colons.

Materials and Methods

Cell Cultures

The Caco-2 cell line was established from a moderately well-differentiated human colon adenocarcinoma [10] and was kindly provided by Dr. J. Fogh (Sloan-Kettering Institute for Cancer Research, Rye, N.Y.). Cells were seeded at 4×10^4 cells/cm^2 and routinely grown at 37°C in either 75 cm^2 plastic flasks or 35 × 10 mm petri dishes containing Dulbecco's modified Eagle medium at 25 mM glucose concentration under a 10% CO_2-90% air atmosphere [31]. The medium was supplemented with 15% fetal bovine serum as suggested by Dr. Fogh (*personal communication*), 1% nonessential amino acids and gentamicin 20 μg/ml. The culture medium was changed daily in all experiments [31]. Cells were used between the 30th and 50th passages. For subculture or cell volume measurement, the confluent cells were dispersed by treatment with 0.05% trypsin and 0.54 mM EDTA and were diluted in the complete culture medium or in the transport medium, respectively. The number, size distribution, and average volume of cells were determined with a pulse height analyzer (Nucleus, Oak Ridge, Tenn.) connected to the output of a Coulter counter.

Enzymatic Characterization of Cultured Cells

Cells were grown in culture flasks for different periods of time. At days 3 and 5, three and two flasks had to be used, respectively, in order to get enough material for enzyme assays. Cells were washed in ice-cold isotonic phosphate-buffered saline (pH 7.4) (PBS), scraped with a rubber policeman and resuspended in 5 ml PBS. Homogenization was performed at 4°C for 1 min at full speed using a Sorvall omnimixer equipped with a micro-attachment device. Enzyme activities were assayed either directly in the homogenates or in the pellet and supernatant fractions issued from 1-hr centrifugation of the homogenates at 100,000 × *g* and 4°C.

Transport Studies on Cell Monolayers

Uptake measurements of AMG were performed with cells attached to the bottom of the petri dishes. The transport medium contained (in mmol/liter): 137 NaCl, 4.7 KCl, 1.2 KH_2PO_4, 1.2 $MgSO_4$, 2.5 $CaCl_2$, 10 HEPES buffer (pH 7.2), 4 glutamine, 0.5 AMG and 0.1 mg/ml bovine serum albumin. When sodium concentrations were varied, NaCl was replaced isosmotically by choline chloride. The osmolarity of each medium was maintained constant at 300 milliosmoles as determined by freezing-point osmometry (Advanced Instrument). Monolayers were washed four times with substrate-free transport medium before incubation at 37°C in 1 ml uptake medium containing 4 μCi of α-methyl-D-(U-^{14}C)glucopyranoside (sp act 150 mCi/mmol). In preliminary experiments, the transport medium also contained 2.5 μCi of either D-(1-^{3}H(N))mannitol (sp act 27.4 Ci/mmol) or (^{3}H(G))inulin (sp act 218.2 mCi/g) for extracellular space corrections. They were later omitted when it appeared that this correction only represented a few percent of accumulated substrate and that it would be included in the residual component of transport measured in the absence of sodium and/or in the presence of 200 μM phlorizin (*see* Table 2 and Fig. 3 in the Results section). Experiments were conducted in the presence or absence of sugar transport inhibitors as indicated in the legends. Inhibitors were prepared as stock solutions in absolute ethanol and diluted to the desired concentrations in the transport medium (final ethanol concentration less than 1%). Control experiments were run using the same concentrations of ethanol. Uptake was terminated by adding 2 ml of ice-cold substrate-free transport medium containing 200 μM phlorizin and 200 μM phloretin. Monolayers were then rinsed four times with this stop solution to get rid of excess radioactivity and solubilized in 0.5 ml NaOH 1 N. Aliquots of 0.3 ml were added to 5 ml Filter Count (United Technologies Packard) for radioactivity determination using a Minaxi Tri-Carb Series 4000, model 4450 scintillation counter (United Technologies Packard) while the remaining was used for protein determination.

Transport Studies on Cell Suspensions

Confluent cells in stationary phase of growth (14 days) were dispersed with 0.05% trypsin and 0.54 mM EDTA, washed in the substrate-free incubation medium described in the preceding section, and harvested by 1-min centrifugation at 1,000 × *g*. Cells were resuspended in the same medium to a final concentration of 4×10^7 cells/ml. Samples of 1 ml were then added to 1 ml incubation medium as to get final conditions similar to those described for transport on monolayers. At time intervals, 0.2 ml aliquots were mixed with 1 ml stop solution (same composition as for monolayers) and the resulting mixture was filtered on prewetted 0.45 μm nitrocellulose filters (Sartorius SM 11306). Filters were washed with 5 ml stop solution, dissolved in mini-vials by 15-min incubation with 5 ml Filter Count and counted as described for monolayers.

Purification of Brush-Border Membrane Vesicles

Confluent cells in stationary phase of growth (14 days) were rinsed twice in ice-cold PBS and either scraped off the flasks using a rubber policeman or dispersed with 0.05% trypsin in 0.54 mM EDTA. In both cases, the cells were washed in PBS and harvested by 1-min centrifugation at 1,000 × *g*. Pellets were then homogenized and brush-border membrane purified by the calcium chloride precipitation method of Schmitz et al. [38] using either 10 [31] or 18 mM [37] $CaCl_2$ as described for Caco-2 cells. Vesicles were obtained by the method of Hopfer et al. [18] with slight modifications as described recently [2].

Human fetal colons were obtained from the Pathology Service of St. Justine Hospital (Montreal, Quebec). For each experiment, 15 to 20 normal fetal colons were pooled. These arrived once or twice a week in groups of 2 to 4. Upon arrival, the lumen was flushed with ice-cold isotonic saline. The mucosa was scraped with a spatula on a cold glass plate, weighted, and frozen at −70°C until use (up to 3 months for the first received colons). Brush-border membrane vesicles were prepared as described above using 10 mM $CaCl_2$ for brush-border membrane purification [2, 18, 38].

Transport Studies on Vesicles

Uptake studies were carried out on brush-border membrane vesicles purified from either 14-day-old confluent cultured cells or human fetal colonic mucosa by the rapid filtration technique of Hopfer et al. [18] as described previously for glutamic acid uptake [2]. Briefly, vesicles were resuspended to a final protein concentration of 5 to 10 mg/ml in 50 mM Tris-HEPES buffer (pH 7.5) containing 375 mM mannitol and 0.1 mM $MgSO_4$. Aliquots (100 μl) were then added to the incubation medium (400 μl) as to

Table 1. Growth-related differentiation of soluble (*S*) and particulate (*P*) brush-border enzymes in Caco-2 cells[a]

Enzyme	Fraction	Days in culture						
		7	10	12	15	19	23	26
	S	*ND*	9.3 ± 1.6	71 ± 6[b]	116 ± 9[b]	97 ± 10	66 ± 13[b]	63 ± 22
Sucrase	*P*	3.3 ± 0.5	80 ± 9[b]	308 ± 29[b]	326 ± 6	311 ± 44	244 ± 48[b]	158 ± 32[b]
			(90 ± 1)	(81 ± 1)[b]	(74 ± 2)[b]	(76 ± 4)	(79 ± 3)	(72 ± 4)
Maltase	*S*	6.8 ± 1.8	62 ± 12[b]	82 ± 8	125 ± 12[b]	110 ± 16	79 ± 10[b]	70 ± 19
	P	30 ± 12	279 ± 14[b]	802 ± 54[b]	939 ± 6[b]	1319 ± 152[b]	859 ± 18[b]	661 ± 42[b]
		(81 ± 3)	(82 ± 3)	(91 ± 1)[b]	(88 ± 1)	(92 ± 1)	(92 ± 1)	(91 ± 1)
Lactase	*S*	5.3 ± 0.3	8.3 ± 1.5	89 ± 9[b]	136 ± 23[b]	100 ± 7[b]		57 ± 7[b]
	P	4.0 ± 1.3	16 ± 3[b]	56 ± 5[b]	78 ± 2[b]	71 ± 5[b]		53 ± 3[b]
		(42 ± 9)	(66 ± 5)[b]	(39 ± 5)[b]	(37 ± 4)	(42 ± 3)		(43 ± 7)
Trehalase	*S*	ND	4.2 ± 0.2	39 ± 3[b]	57 ± 10[b]	45 ± 5[b]	21 ± 5[b]	24 ± 4
	P	2.2 ± 0.5	9.6 ± 0.3	113 ± 9[b]	38 ± 2[b]	35 ± 2	23 ± 1[b]	26 ± 2
Amino			(71 ± 3)	(75 ± 1)	(40 ± 3)[b]	(44 ± 4)	(52 ± 5)[b]	(52 ± 4)
peptidase	*S*	9 ± 3	38 ± 7[b]	60 ± 2[b]	65 ± 7	50 ± 8	80 ± 14[b]	63 ± 6[b]
	P	18 ± 5	31 ± 8	40 ± 5	73 ± 6[b]	93 ± 11[b]	113 ± 14[b]	77 ± 16[b]
		(68 ± 2)	(42 ± 2)[b]	(40 ± 4)	(53 ± 3)[b]	(65 ± 1)[b]	(58 ± 5)[b]	(59 ± 1)

[a] Values shown are the mean ± SD of enzyme activities (mU/flask) for three different subcultures. Values in brackets represent the % enzyme activity recovered in the *P* fraction. [b] Indicates significant differences ($P < 0.05$) between consecutive days in culture. ND: not detectable.

get final concentrations of 50 mM Tris-HEPES buffer (pH 7.5), 0.1 mM $MgSO_4$, 75 mM mannitol, 150 mM NaCl or 150 mM KCl, 0.1 mM D-(U-^{14}C)glucose (sp act 350 mCi/mmol), 1% absolute ethanol, and either 200 μM phlorizin or 1 mM phloretin or 10 mM AMG or 10 mM leucine. Uptake was conducted at room temperature (20°C) and, at time intervals, aliquots (50 μl) were sampled from the incubation mixture, quenched in 1 ml ice-cold stop-solution containing 200 μM phlorizin, filtered on prewetted 0.45 μm nitrocellulose filters and washed with 4 ml stop solution. Filters were dissolved and counted as described for suspended cells.

Assays

Disaccharidase activities (maltase, lactase, sucrase, trehalase) were assayed by the method of Dahlqvist [6] as modified by Lloyd and Whelan [22]. γ-Glutamyltransferase activity was assayed by the method of Naftalin et al. [29]. Leucylnaphthylamide-hydrolyzing and alkaline phosphatase activities were assayed according to Goldbarg and Rutenburg [12] and Eichholz [7], respectively. Na^+,K^+-ATPase was assayed as described by Post and Sen [32] and the phosphate liberated during the reaction by the method of Fiske and Subbarow [9]. Protein was assayed according to Lowry et al. [23] using crystalline bovine serum albumin as standard.

Presentation of Data

Enzyme activities are expressed as milliunits per mg of protein: one unit is defined as the activity which hydrolyzes 1 μmol of substrate per minute under the experimental conditions. Intracellular accumulation of AMG is expressed as nmol solute uptake per mg protein. Unidirectional influx was estimated by either linear regression of accumulation time courses over the period 0 to 60 min (nmol per mg protein per min) or by one time-point analysis (nmol per mg protein per 30 min) as indicated in the legends.

Values shown in Figures and Tables are the mean ± SD for at least three different experiments with cells from different passages. Statistical analyses were performed with Statcalc [21] using an Apple IIe microcomputer. Comparisons were done by one-way analysis of variance and significant differences were estimated from the drawn Bonferroni confidence intervals ($P < 0.05$).

Materials

Cell culture media and plastic wares (Falcon) were obtained from Gibco Laboratories and Becton Dickinson Labware, respectively. Radioisotopes were purchased from New England Nuclear (D-mannitol, D-glucose, inulin) or Amersham (AMG). Phlorizin, phloretin and ouabain were obtained from Sigma Chemical Co. All other chemicals were of the highest purity available.

Results

Enzymatic Characterization of Cultured Cells

The growth-related appearance of different enzymes which have been specifically localized to the brush-border membrane of the functional enterocyte [24, 38] is shown in Table 1. Enzyme activities were assayed in supernatant (*S*) and pellet (*P*) fractions issued from 1-hr centrifugation of cell homogenates at 100,000 × *g*. Only alkaline phosphatase and γ-glutamyltransferase were entirely recovered

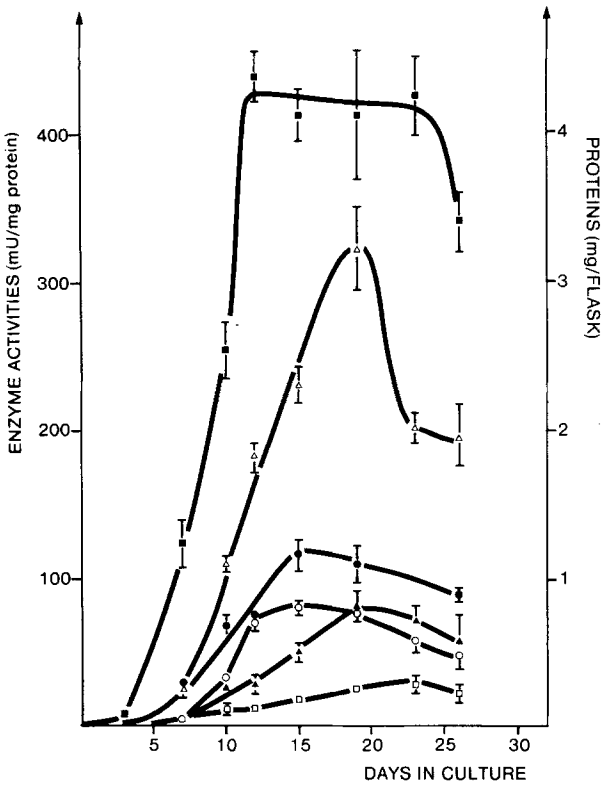

Fig. 1. Growth-related differentiation of brush-border membrane enzymes in Caco-2 cells. Proteins (■) and enzymes were assayed in the membrane fraction as described in the text. Values are the mean ± SD evaluated on three different subcultures for maltase (△), alkaline phosphatase (●), sucrase (○), γ-glutamyltransferase (▲), and aminopeptidase (□)

in the particulate fraction (*not shown* for this reason) while Table 1 shows that partition between soluble and membrane forms of the enzymes varied from one species to the other. In stationary cultures (15 to 23 days), maltase, sucrase and aminopeptidase were mostly found associated with the membrane fraction (88–92, 74–79, and 53–65%, respectively) while other disaccharidases, lactase and trehalase, were mostly soluble (37–42 and 40–52%, respectively). It also appears from Table 1 that the proportion of enzyme activities recovered in the membrane fraction is not constant during the culture. In general, higher membrane-associated activities preceded or paralleled the peak activity in the particulate fraction.

The data presented in Fig. 1 correlate the enzyme specific activities found in the membrane fraction of cell homogenates with the cell growth characteristics. This last parameter is well described by the quantity of proteins per flask recovered in the particulate fraction which closely followed the cell number per flask (*not shown*) and represented a constant proportion of total cell proteins along the culture (45 ± 5%, mean ± SD of the 24 time points of this experiment). The protein curve in Fig. 1 (closed squares) shows that proliferation started by day 3 to reach a stationary phase by day 12. However, the protein content decreased by day 23, a time at which the monolayer began to detach from the edges of the flasks. It should, however, be noted that confluency was reached by day 7 or 8 as evidenced by the presence of domes on the monolayer,

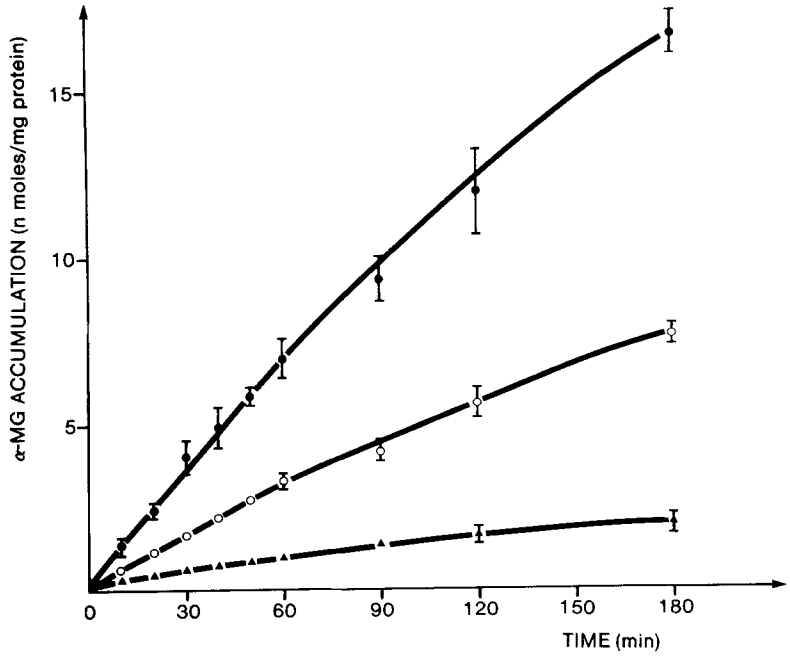

Fig. 2. Time course of 0.5 mM AMG accumulation in Caco-2 cells. Sugar accumulation was performed as described in the text in the absence of inhibitors (●) and in the presence of 200 μM phlorizin (▲) or phloretin (○). Points shown are the mean ± SD evaluated on three different subcultures. It should be noted that linearity is respected up to 60-min incubation (coefficient of correlation > 0.99 in the three cases)

in accordance with previous reports [15, 31]. Figure 1 also reveals that peak activities in the cell membrane fraction varied for the different enzymes considered. Trehalase (*not shown*, but *see* Table 1) peaked first at day 12, followed by sucrase (open circles), lactase (*not shown*, but *see* Table 1) and alkaline phosphatase (closed circles) at day 15, by maltase (open triangles) and γ-glutamyltransferase (closed triangles) at day 19, and by aminopeptidase (open squares) at day 23.

AMG Accumulation in Fourteen-Day-Old Monolayers of Caco-2 Cells

Figure 2 shows the time course of AMG accumulation in 14-day-old confluent Caco-2 cell monolayers. In the absence of inhibitors (upper curve), it is readily apparent that the rate of sugar uptake proceeded linearly up to 60-min incubation but decreased thereafter. Moreover, steady-state distribution of tracer was not reached even after 3-hr incubation. Figure 2 also shows that AMG accumulation was considerably depressed by phlorizin addition to the incubation medium (lower curve). A similar inhibition of AMG transport was also obtained after isotonic substitution of NaCl by choline chloride (Table 2). With an average cell volume of 3.66 ± 0.20 μl/mg protein (mean ± SD for seven determinations at day 14), ratios of intracellular-to-extracellular concentrations of AMG can be estimated and values of 3.80 ± 0.35 and 9.13 ± 0.35 were obtained after 1- and 3-hr incubation in the absence of inhibitor, respectively. In the presence of phlorizin, ratios of 0.52 ± 0.04 and 1.07 ± 0.18 can be calculated at each corresponding time and it thus appears from Table 2 that active AMG transport was completely abolished by either phlorizin addition or complete sodium replacement.

In order to delineate more closely the pathways for AMG transport, we also have analyzed the effect of phloretin on sugar accumulation. Figure 2 shows that a 200-μM concentration of this inhibitor (middle curve) produced a partial decrease in both the initial rate of uptake and the overall accumulation of sugar. Active AMG transport was still observed in these conditions as accumulation ratios of 1.80 ± 0.14 and 4.22 ± 0.21 can be estimated after 1- and 3-hr incubation, respectively. Finally, it appears from Table 2 that the combined addition of phlorizin and phloretin to the incubation medium did not produce any further inhibition as compared to the maximum inhibition produced by phlorizin alone or to Na^+-free conditions. It thus seems appropriate to define the baseline value (simple diffusion) by the mean of these last three experimental conditions (Table 2). After correction for this value, a 60.7% inhibition by phloretin of Na^+-dependent AMG transport can be determined (Table 2).

The sensitivity to phlorizin and phloretin was further documented by studying the effect of different concentrations of the two inhibitors on the initial rates of AMG accumulation and by comparing it with the inhibition produced by addition of cold D-glucose to the incubation medium. The results of this study are shown in Fig. 3 and it clearly appears that both D-glucose and sugar transport inhibitors could produce complete inhibition of sugar accumulation. However, different I_{50} were found for these three substances. On a molar basis, phlorizin is the more potent inhibitor followed by phloretin and then D-glucose.

Table 2. Unidirectional rates of AMG accumulation in Caco-2 cells in different experimental conditions[a]

Experimental conditions	Initial rates of α-MG accumulation (nmol/mg protein min)	Inhibition (%)	Inhibition of Na^+-dependent flux (%)
NaCl (n = 6)	0.1136 ± 0.0090	—	—
NaCl + phloretin (n = 4)	0.0525 ± 0.0037[b]	53.8	60.7
NaCl + phlorizin (n = 5)	0.0125 ± 0.0016[b,c,d]	89.0	100
NaCl + phloretin + phlorizin (n = 4)	0.0126 ± 0.0003[b,c,d]	88.9	100
Choline chloride (n = 2)	0.0130 ± 0.0008[b,c,d]	88.6	100

[a] Initial rates were estimated by linear regression of accumulation time courses over the time period 0 to 60 min. Values shown are the mean ± SD of n determinations on different subcultures. [b] and [c] indicate significant differences ($P < 0.05$) relative to NaCl and NaCl + phloretin, respectively. [d] indicates values which are not significantly different ($P > 0.05$).

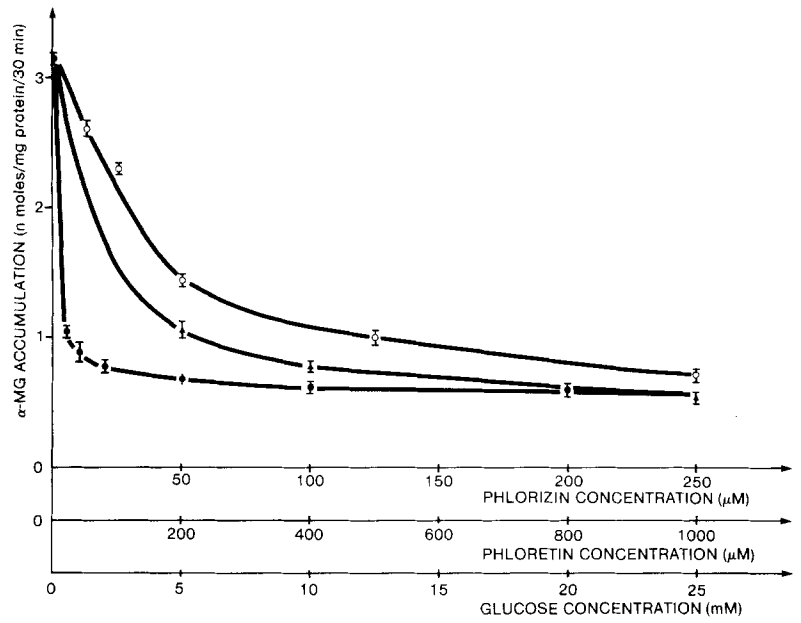

Fig. 3. Inhibition of 0.5 mM AMG influx in Caco-2 cells as a function of D-glucose (▲), phlorizin (●) and phloretin (○) concentrations. Mean unidirectional influxes ± SD were estimated by one time-point analysis (30 min) on three different subcultures

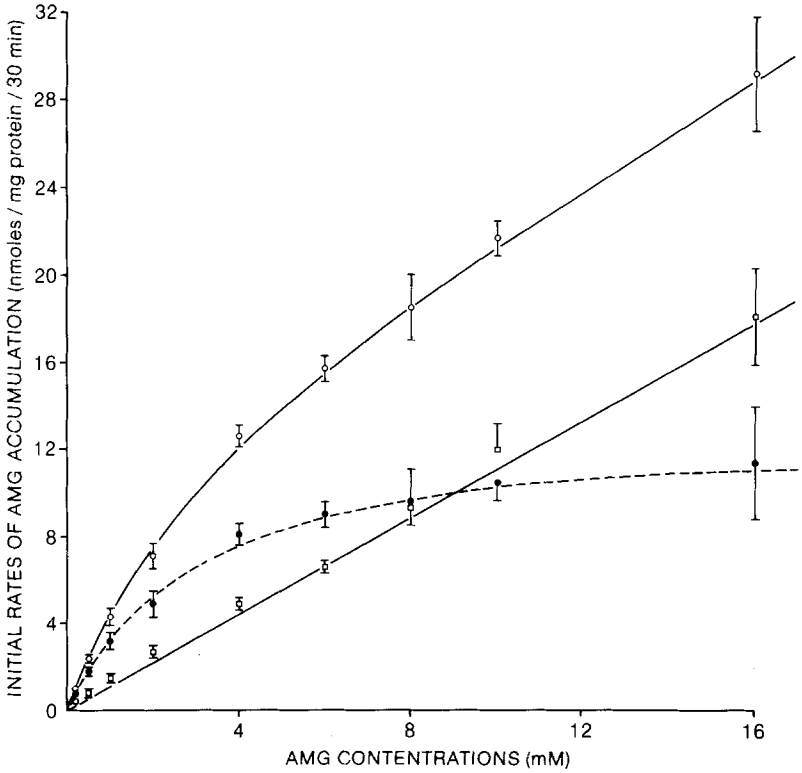

Fig. 4. Kinetics of AMG transport in Caco-2 cells. Initial rates of AMG accumulation were estimated by one time-point analysis (30 min) as a function of substrate concentrations in the absence of inhibitors (○) or in the presence of 1 mM phlorizin (□). Total accumulation corrected for the diffusional component is also shown (●). Values are the mean ± SD of three to nine determinations on one to three different subcultures. Drawn lines correspond to the best fit of the data according to values of parameters shown in the text

Kinetics of AMG Transport in Fourteen-Day-Old Monolayers of Caco-2 Cells

As linearity with incubation time was observed up to 60 min, the kinetics of AMG accumulation were analyzed by one time-point analysis (30 min) over the range of substrate concentrations 0.2 to 16 mM. The results of this study are presented in Fig. 4 where it can be appreciated that total AMG transport (upper curve) did not saturate over the range studied. When AMG transport was recorded in the presence of 1 mM phlorizin, the lower curve was obtained which could be fitted to a straight line (correlation coefficient 0.99). This component of AMG transport is thus compatible with a simple diffusive pathway with a K_d of 1.11 ± 0.03 nmol/mg protein/30 min/mM. This value agrees well with the accu-

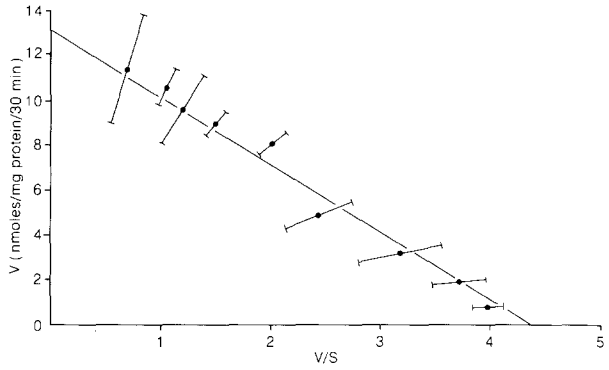

Fig. 5. Hofstee plot of AMG transport in Caco-2 cells. Conditions were as in Fig. 4 and regression was performed using Statcalc on the data points obtained after correction for the diffusional component of transport

mulation of 0.57 ± 0.06 nmol/mg protein of AMG as recorded after 30-min incubation in the presence of phlorizin in the conditions of Fig. 2. When this diffusive component was subtracted from the total uptake, it then appeared that saturation of AMG transport could be observed (Fig. 4, middle curve). This saturating component of AMG transport was found to linearize (correlation coefficient 0.89) according to Hofstee analysis (Fig. 5) and thus is compatible with a single transport system corresponding to Michaelis-Menten kinetics with K_m and V_m of 3.1 ± 0.2 mM and 13.2 ± 0.5 nmol/mg protein/30 min, respectively. It can also be appreciated that the data from Fig. 4 were satisfactorily fitted according to above parameter values (lines shown correspond to calculated values).

Uptake Studies in Brush-Border Membrane Vesicles Isolated from Caco-2 Cells and in Cell Suspensions of Caco-2 Cells

The membrane localization of the Na^+-dependent sugar transport pathway present in Caco-2 cells can theoretically be determined directly using membrane vesicles [18]. However, our attempts to demonstrate D-glucose transport in brush-border membrane vesicles isolated from Caco-2 cells have failed so far. By the techniques described under Materials and Methods, it, however, appeared that brush-border membrane purifications were successful (sucrase enrichment factor of 15- to 17-fold over the starting homogenate). As the first step in the isolation procedure involved cell harvesting by either scraping with a rubber policeman or trypsin-EDTA treatment, experiments were conducted in order to test transport functions in cell suspensions obtained by both procedures. In both cases, AMG accumulation could be recorded for up to 5 min of incubation

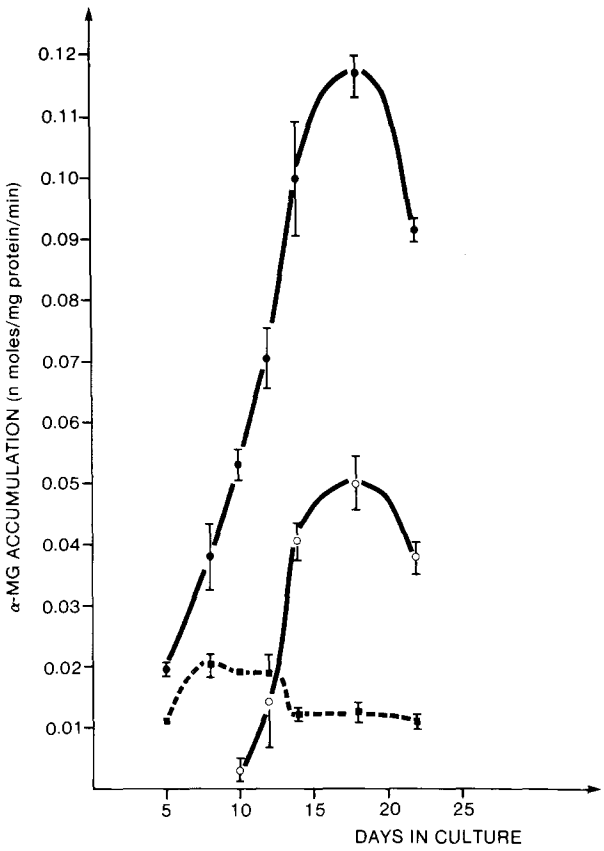

Fig. 6. Growth-related differentiation of AMG transport in Caco-2 cells. Unidirectional influxes of 0.5 mM AMG were estimated by linear regression of accumulation time courses over the time period 0 to 60 min. Values shown in the absence of inhibitor (●) and the presence of 200 μM phloretin (○) have been corrected for the diffusional component measured in the presence of 1 mM phlorizin (■) and represent the mean ± SD of three determinations on different subcultures

with the radioactive substrate but collapsed very rapidly thereafter (*results not shown*). Thus, sustained transport activities cannot be obtained in these conditions.

Growth-Related Differentiation of AMG Transport

The membrane localization of the Na^+-dependent transport pathway for AMG accumulation in confluent Caco-2 cells was indirectly analyzed by following the kinetics of evolution of both the phlorizin- and phloretin-sensitive unidirectional influxes during the culture. The results of this experiment are presented in Fig. 6 where it can be appreciated that the two transport activities (corrected for the diffusional component) appeared synchronously during the differentiation process with a maximum around day 18 and a small decrease thereafter. However, the sensitivity to 200 μM phloretin decreased from 94% at day 10 to 57–59% at day 14 and later (Table

3). Figure 6 also shows that the diffusive pathway for AMG transport was quite stable during the culture although decreasing by 39% from day 8–12 to day 14–22. Finally, the comparison between Fig. 6 and Fig. 1 clearly indicates that the phlorizin- and phloretin-sensitive pathway for sugar accumulation closely followed the growth-related differentiation of brush-border membrane enzyme activities.

Energetics of AMG Accumulation in Fourteen-Day-Old Monolayers of Caco-2 Cells

The membrane localization of the Na^+-dependent pathway for AMG accumulation was further analyzed by comparing the kinetics of transport inhibition by phlorizin and phloretin with those by ouabain. In the experiments presented in Fig. 7, AMG accumulation was followed for up to 1 hr in the absence of inhibitors. Phlorizin, phloretin and ouabain were then added to different petri dishes and their effects followed during the next 3 hr. When compared to the control situation (no inhibitor added over the same time period), it is readily apparent from Fig. 7 that ouabain (open circles) and phloretin (open triangles) decreased the rate of AMG accumulation while phlorizin (closed triangles) completely inhibited it. However, as compared to ouabain, phloretin produced a greater inhibition on the influx rate but never produced complete inhibition of sugar accumulation over the time period studied. The effect of higher concentrations of phloretin (1 mM) could not be tested and compared to phlorizin as such conditions induced cell detachment from the petri dishes for incubation periods in excess of 30 min. The low rates of AMG efflux following complete inhibition by phlorizin should also be noted in Fig. 7.

Table 3. Fraction of Na^+-dependent AMG influx inhibited by 200 μM phloretin during Caco-2 cell differentiation[a]

Days in culture	Inhibition of α-MG accumulation by 200 μM phloretin (%)
10 (n = 3)	94.0 ± 3.5
12 (n = 3)	80.9 ± 9.2[b]
14 (n = 4)	58.8 ± 6.8[b,c]
18 (n = 3)	57.1 ± 4.4[c]
22 (n = 3)	58.6 ± 2.3[c]

[a] Values were derived from the data shown in Fig. 6 and represent the mean ± SD of n determinations. [b] and [c] indicate significant differences ($P < 0.05$) between consecutive days and values which are not significantly different ($P > 0.05$), respectively.

Glucose Uptake by Brush-Border Membrane Vesicles Isolated from the Human Fetal Colon

As demonstrated by others, cancerous Caco-2 cells present some functional properties which are normally associated with colon cells of human fetuses

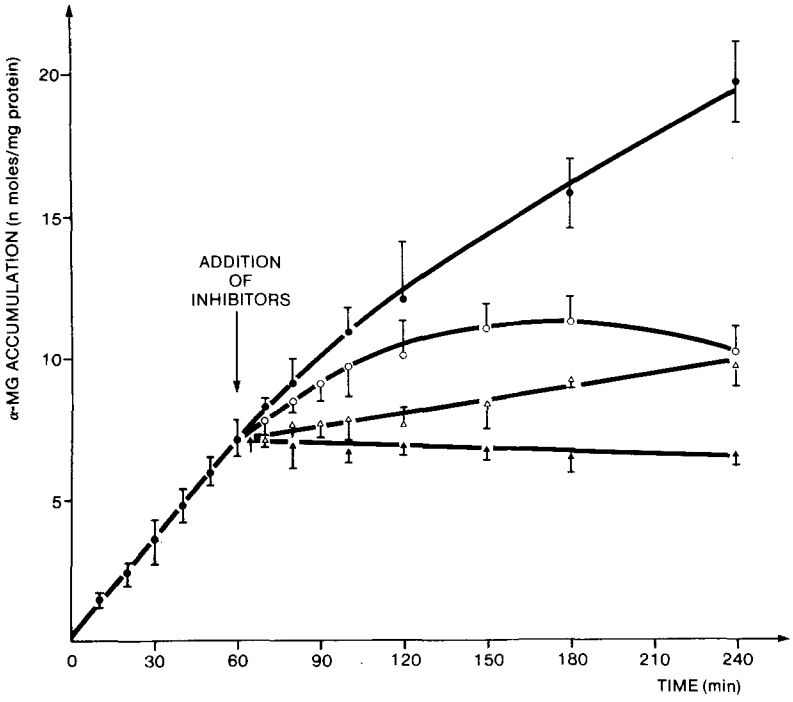

Fig. 7. Inhibitor-induced alterations in AMG accumulation by Caco-2 cells. Phlorizin (▲), phloretin (△), and ouabain (○) at concentrations of 200 μM, or equivalent ethanolic solution (●) were added 1 hr after initial exposure of the cells to 0.5 mM AMG as indicated by the arrow. Values shown are the mean ± SD for three determinations on different subcultures

under 24 weeks of age [31, 43]. If true, the human fetal colon should exhibit (a) sugar transport pathway(s) with characteristics similar to those found in Caco-2 cells. This hypothesis was thus tested using brush-border membrane vesicles isolated from the human fetal colonic mucosa. Two experiments were carried out under identical conditions and with identical results. One experiment is reported in Fig. 8, which allows the following conclusions to be drawn. First, the presence of a Na^+-gradient (outside > inside) stimulated D-glucose uptake (upper line, closed circles) as compared to a K^+ gradient (lower line, open circles), thus showing the Na^+-dependency of uptake. However, a transient accumulation over equilibrium uptake values (overshoot) was hardly detectable in these vesicles. Next, introducing either phlorizin (200 μM) or phloretin (1 mM) in the incubation medium completely abolished the Na^+-dependent stimulation of uptake (lower curve, closed and open triangles for phlorizin and phloretin, respectively), thus showing complete inhibition of Na^+-dependent transport by both inhibitors. Finally, the Na^+-dependent uptake of D-glucose was completely inhibited by addition of 10 mM AMG in the incubation medium (lower curve, closed squares) while completely insensitive to the same concentration of L-leucine (upper curve, open squares), thus showing the specificity for a sugar transport pathway and ruling out substrate competition for the driving force (electrochemical Na^+-gradient). It should also be noted that the curves drawn in Fig. 8 extrapolated through the origin and that similar equilibrium values were obtained in all experimental conditions, thus showing the absence of binding to the membranes and of membrane disruption by any of the tested substances, respectively.

The last question to be answered concerned the purity of the vesicle preparations used in these studies. This was addressed by assaying sucrase and Na^+,K^+-ATPase in both homogenates and vesicle fractions in order to evaluate the enrichment in brush-border and basolateral membranes, respectively. It was found that the brush-border mem-

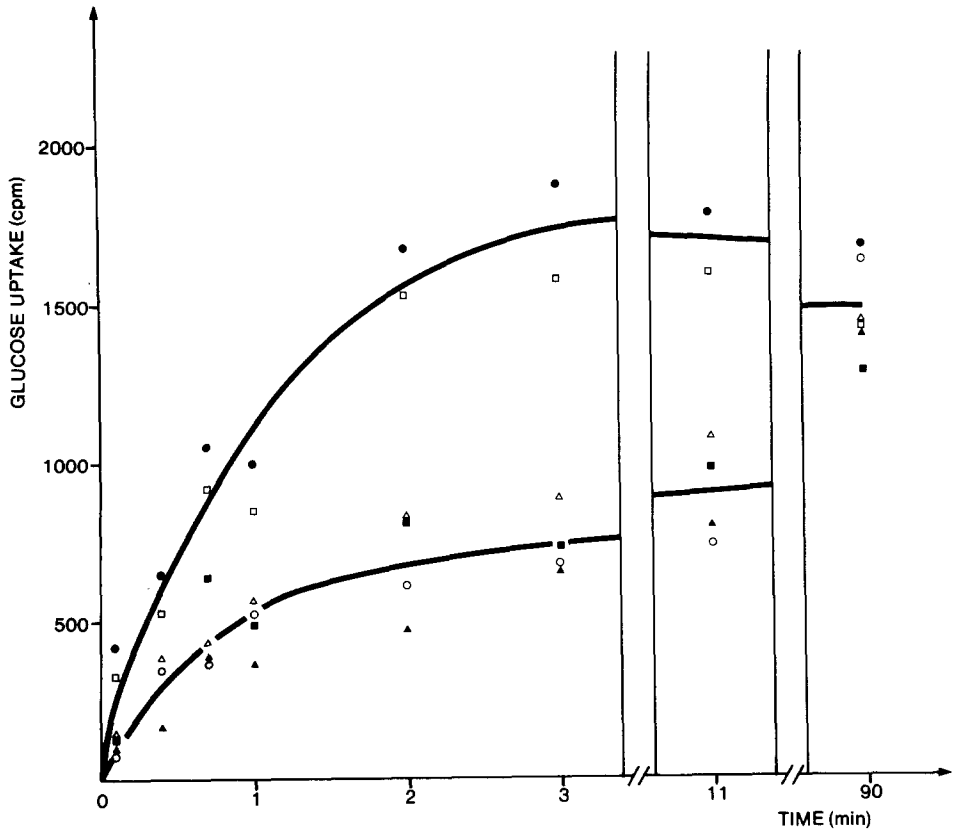

Fig. 8. Glucose uptake by brush-border membrane vesicles isolated from the human fetal colonic mucosa. Uptake of 0.1 mM D-glucose was analyzed in sodium-gradient conditions (150 mM) in the absence (●) or presence of 200 μM phlorizin (▲), 1 mM phloretin (△), 10 mM AMG (■), 10 mM L-leucine (□), and compared to similar potassium gradient conditions (○). Curves shown have been obtained by polynomial fitting of the data points. Statistical analysis could not resolve the lower curve in more than one component. Values shown are from a typical experiment out of two performed under identical conditions

brane marker sucrase purified 20-fold over the starting homogenate while the basolateral marker Na^+,K^+-ATPase showed a negative enrichment of 0.6-fold.

Discussion

In their first report on the enterocyte-like differentiation and polarization of Caco-2 cells in culture, Pinto et al. [31] have measured growth-related activities of alkaline phosphatase, sucrase and aminopeptidase after partial purification of brush-border membranes. Though clearly showing the synchronism between morphological and functional differentiation of the brush-border membrane, this procedure may, however, have masked the real evolution of enzyme activities during culture. For example, enzymic proteins may have been synthesized before confluency and thus not been accounted for after isolation of brush-border membranes. Also, the varying composition of the brush-border membrane during the differentiation process [31] may have been accompanied by changes in membrane sensitivity to calcium, thus leading to different yields in the membrane fraction as function of time in culture. To circumvent these problems, we have analyzed enzyme activities in supernatant (soluble) and pellet (membrane) fractions issued from 1-hr centrifugation at 100,000 × *g* of cell homogenates. The work of Pinto et al. [31] was also extended to include other known brush-border membrane enzymes like maltase, lactase, trehalase and γ-glutamyltransferase [24, 38].

It first appears that alkaline phosphatase and γ-glutamyltransferase were the only two enzyme activities found exclusively associated with the membrane fraction. The other enzyme activities partitioned to different degrees between particulate and soluble fractions according to enzyme species and time in culture (Table 1). It should be noted that soluble forms of brush-border membrane enzymes have already been reported in large amounts in the neonatal small intestine [11, 39, 41] and during organ culture of the small intestine [3]. They have also been characterized in intestinal tissues and found to present some differences with their brush-border counterparts [4, 25]. However, their origin and role are only speculative [3, 4, 25, 41].

It also appears that the membrane-bound enzymes have their own pattern of differentiation (Fig. 1), in agreement with previous studies in organ culture [3, 8, 17, 26] and the demonstration for independent development [13, 28], regulation [34] and biosynthesis [16, 30] of brush-border enzymes. The only common characteristics for all enzyme species are their almost complete absence and their maximum expression before day 7 and between days 12 to 23 in culture, respectively. The functional differentiation of Caco-2 cells is thus a growth-related phenomenon. The apparition of brush-border membrane enzymes is associated with the polarization of the epithelial monolayer (as evidenced by dome formation around the 7th day in culture) and their maturation is achieved during the stationary phase of growth (as evidenced by constant protein recovery per culture flask, Fig. 1). These results thus agree with the previously reported growth characteristics of Caco-2 cells and the timing for functional differentiation of the brush-border membrane during culture [31].

The question as to whether Caco-2 cells would exhibit Na^+-coupled transport of solutes has been answered positively for alanine [15] and inorganic phosphate [27] but negatively for glucose [15]. This last result was reinvestigated in this paper using isotopic tracer flux measurements of AMG. Our results clearly demonstrate the presence of both Na^+-independent and Na^+-dependent pathways for sugar transport in Caco-2 cells. The first one is likely to represent simple diffusion as shown by kinetic (Fig. 4) and inhibition (Table 2 and Fig. 3) studies. The unicity, membrane localization and nature of the second one are, however, more difficult to ascertain as this component was found to be inhibited by both phlorizin and phloretin but, in any case, its presence in Caco-2 cells is a conclusion at variance with that of Grasset et al. [15]. This apparent discrepancy could be resolved by postulating a slowly functioning carrier which, for this reason, is not detectable by electrical parameter measurements [15]. Such an explanation would be consistent with the observation made by these authors [15] that 25 mM alanine only produced a very small, although statistically significant, increase in short-circuit current, close in fact to the lower limit of the sensitivity of the measurement method.

Phloretin sensitivity of glucose transport has usually been associated with the presence of a Na^+-independent, facilitated diffusion pathway in the basolateral membranes of mature enterocytes [19]. The existence of such a pathway for AMG transport in Caco-2 cells is, however, ruled out by the following observations. First, inhibition by Na replacement or phlorizin (Table 2) was identical with that by phloretin or D-glucose (Fig. 3). Next, phloretin inhibition was not additive to the maximum inhibition that was achieved with phlorizin alone (Table 2). Finally, the Na^+-independent, phlorizin-insensitive component of AMG transport behaves as a simple diffusive process as shown by the lack of both intracellular substrate accumulation (Fig. 2) and

saturation with substrate concentrations (Fig. 4). It must then be concluded that AMG transport in Caco-2 cells is specific to a Na^+-dependent pathway(s) as was found in chicken enterocytes [20]. This conclusion agrees with the low efflux rates that were recorded after inhibition by phlorizin (Fig. 7) but does not rule out the existence of a Na^+-independent, phloretin-sensitive pathway in Caco-2 cells. Other substrates are now being tested in search of such a pathway and preliminary results indicate that 2-deoxyglucose can be successfully employed for this purpose.

The presence in Caco-2 cells of a single, Na^+-dependent AMG transport system which is sensitive to both phlorizin and phloretin is compatible with the following observations. First, complete inhibition of AMG accumulation was achieved by high concentrations of D-glucose, phlorizin, and phloretin (Fig. 3). Next, kinetic studies are compatible with the presence of only one transport system for the phlorizin-sensitive component of AMG unidirectional influx (Figs. 4 and 5). Finally, the growth-related functional differentiation of Na^+-dependent AMG transport was identical for the phlorizin- and phloretin-sensitive components (Fig. 6). However, the sensitivity to phloretin was also related to cell growth as the percent inhibition by a suboptimal concentration of phloretin decreased from day 10 to days 14 to 22 (Table 3). Although this behavior could reflect heterogeneity in sugar transport functions in the early phase of the differentiation process, it could as well be interpreted as being part of the maturation process of the Na^+-dependent carrier. More studies will be needed to clarify this point.

The brush-border membrane localization of the Na^+-dependent AMG transport system observed in Caco-2 cells would be compatible with the known properties of this transport system in mature enterocytes (Na^+-dependency, phlorizin-sensitivity) [40] and its substrate specificity for AMG [20]. Such a view is strengthened by the immediate inhibition of AMG uptake induced by phlorizin and phloretin addition 1 hr after initiating sugar transport (Fig. 7). Moreover, a toxic effect of phloretin that would deplete the ATP energy stores and consequently decrease the sodium electrochemical gradient available for transport can be ruled out when considering the sustained increase in AMG accumulation after phloretin addition. This behavior also contrasts with the complex inhibition pattern produced by ouabain addition (Fig. 7) that must be the consequence of a progressive loss in the energy available for active transport through the dissipation of the Na^+-electrochemical gradient that follows inhibition of Na^+,K^+-ATPase by ouabain in these cells [15, 35]. This comparison thus rules out a possible action of phloretin through inhibition of the Na^+,K^+-ATPase.

The brush-border membrane localization of the Na^+-dependent AMG transport system in Caco-2 cells is also compatible with the observation that the functional differentiation of AMG transport (Fig. 6) was synchronous with the development of brush-border membrane enzyme activities (Fig. 1). This behavior is quite different from the development pattern reported for Na^+-dependent transport of phosphate in these cells [27] which showed decreased activity from plating to confluency and minimal activity during the stationary phase of growth (*see* Fig. 4 in [27]). It thus appears that the apical phosphate carrier demonstrated with brush-border membrane vesicles isolated from Caco-2 cells may not represent the only and major Na^+-dependent pathway in these cells before and even after confluency. Our own attempts to demonstrate the presence of a Na^+-dependent, phlorizin- and phloretin-sensitive transport pathway for D-glucose in brush-border membrane vesicles isolated from confluent Caco-2 cells have failed so far. This negative result could argue against the apical localization of this transport system as discussed above. We, however, do not think that this is necessarily the case as suspensions of isolated cells also failed to show sustained AMG accumulation for time periods longer than 5-min incubation. It thus appears that carrier stability in the membrane, whatever its membrane localization, would represent instead the major problem in these studies. Whether this instability is the result of cell polarity and/or cell contact disruption or sensitivity to trypsin and/or EDTA remains to be answered. This is in contrast to the situation demonstrated in our experiments and those of other laboratories [27, 31, 37] for brush-border membrane enzyme activities which survived both monolayer disruption and membrane purification. This is also at variance with the results reported for phosphate transport in Caco-2 cells [27]. We have no explanation for these differences but vesicle leakiness and/or different turnover rates between brush-border membrane components are possibilities that should be considered.

The hypothesis that cancerous Caco-2 cells may present some functional properties normally associated with fetal colonic cells [31, 43] was investigated further by analyzing Na^+-dependent D-glucose transport in brush-border membrane vesicles isolated from the human fetal colon. This preparation showed good enrichment in sucrase activity and low contamination by the basolateral marker Na^+,K^+-ATPase. The functional characteristics of D-glucose transport determined in these

vesicles would thus represent those prevailing in vivo at the brush-border membrane level in the human fetal mucosa. Our studies clearly demonstrate the presence of a Na^+-dependent component for D-glucose uptake, which is completely inhibited by both phlorizin and phloretin (Fig. 8). D-glucose uptake was also inhibited to diffusion levels by 10 mM AMG but was insensitive to the presence of leucine at the same concentration (Fig. 8). All together, these data indicate similar functional characteristics for Na^+-sugar cotransport in brush-border membranes of the human fetal colon and Caco-2 cells in culture. To our knowledge, this is the first demonstration of Na^+-dependent sugar transport by the human fetal colon. This finding agrees with the known enterocyte-like properties of this tissue during the middle trimester of fetal life [13] and the recent report that the colon of 20-day-old fetal rats demonstrates Na^+-dependent, phlorizin-sensitive transport of D-glucose [33].

Whether Caco-2 cells may represent mature enterocytes [31], colonic crypt cells [14, 15] or fetal colonic cells [31, 37, 43] is still a matter of controversy. This issue cannot be resolved by our studies as phloretin inhibition of Na^+-dependent sugar transport system(s) demonstrated in Caco-2 and human fetal colonic cells may not represent a decisive criterion as to the nature of the transport system(s) involved. In this context, it should be noted that phloretin sensitivity of Na^+-dependent sugar transport in the adult small intestine was reported in hamster [1, 5] and rabbit [42] but not in chicken [20] and, to our knowledge, was never assessed in humans. Kinetic analysis have shown that phlorizin, a fully competitive and phloretin, a fully noncompetitive inhibitor compete mutually for the transport system [5, 41]. These observations thus suggested common step(s) in their mechanisms of inhibition and sugar- and phenol-binding sites on the carrier have been postulated [1, 5, 41]. However, it also appears that phloretin only inhibited Na^+-energized D-glucose uptake in the rabbit, so that phloretin may not interact directly with the transporter *per se* [41]. Whatever the exact molecular mechanism for phloretin inhibition of the apical, Na^+-dependent sugar transport system, it thus seems that phloretin inhibition could well represent a more general phenomenon than usually recognized and that it may vary according to the animal species considered [1, 20]. It follows from these considerations that phloretin inhibition of Na^+-dependent sugar transport does not represent a discriminating criterion as to the nature and membrane localization of this system. Work is now under progress in our laboratory to determine the mechanism of inhibition of Na^+-dependent AMG transport by phlorizin and phloretin in Caco-2 cells and to test for the phloretin sensitivity of Na^+-dependent sugar transport in the adult human small intestine.

In summary, we have demonstrated the presence in Caco-2 cells of a Na^+-dependent sugar transport system with properties similar to those normally found in brush-border membranes of 16- to 22-week-old human fetal colon. Caco-2 cells thus appear as a valuable model to study the mechanisms involved in the differentiation and regulation of intestinal transport functions.

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References

1. Alvarado, F. 1967. Hypothesis for the interaction of phlorizin and phloretin with membrane carriers for sugars. *Biochim. Biophys. Acta* **135**:483–495
2. Berteloot, A. 1984. Characterization of glutamic acid transport by rabbit intestinal brush-border membrane vesicles. Effect of Na^+-, K^+- and H^+-gradients. *Biochim. Biophys. Acta* **775**:129–140
3. Berteloot, A., Chabot, J.G., Hugon, J.S. 1981. Turnover of mouse intestinal brush border membrane proteins and enzymes in organ culture. A direct evaluation from studies on the evolution of enzyme activities during the culture. *Biochim. Biophys. Acta* **678**:423–436
4. Cezard, J.P., Conklin, K.A., Das, B.C., Gray, G.M. 1979. Incomplete intracellular forms of intestinal surface membrane sucrase-isomaltase. *J. Biol. Chem.* **254**:8969–8975
5. Colombo, V.E., Semenza, G. 1972. An example of mutual competition between transport inhibitors of different kinetic type: The inhibition of intestinal transport of glucalogues by phloretin and phlorizin. *Biochim. Biophys. Acta* **288**:145–152
6. Dahlqvist, A. 1964. Method for assay of intestinal disaccharidases. *Anal. Biochem.* **7**:18–25
7. Eichholz, A. 1967. Structural and functional organization of the brush border of intestinal epithelial cells. III. Enzymic activities and chemical composition of various fractions of tris-disrupted brush borders. *Biochim. Biophys. Acta* **135**:475–482
8. Falchuk, Z.M., Gebhard, R.L., Sessons, C., Strober, W. 1974. An in vitro model of glutensensitive enteropathy: Effect of gliadin on intestinal epithelial cells of patients with glutensensitive enteropathy in organ culture. *J. Clin. Invest.* **53**:487–500
9. Fiske, C.H., Subbarow, Y. 1925. The colorimetric determination of phosphorus. *J. Biol. Chem.* **66**:375–400
10. Fogh, J., Fogh, J.M., Orfeo, T. 1977. One hundred and twenty seven cultured human tumor cell lines producing tumors in nude mice. *J. Natl. Canc. Inst.* **59**:221–226
11. Galand, G., Forstner, G.G. 1974. Isolation of microvillus plasma membranes from suckling-rat intestine. The influence of premature induction of digestive enzymes by injection of cortisol acetate. *Biochem. J.* **144**:293–302

12. Goldbarg, J.A., Rutenburg, A.M. 1958. The colorimetric determination of leucine aminopeptidase in urine and serum of normal subjects and patients with cancer and other diseases. *Cancer* **11**:283–291
13. Grand, R.J., Watkins, J.B., Torti, F.M. 1976. Development of the human gastrointestinal tract. A review. *Gastroenterology* **70**:790–810
14. Grasset, E., Bernabeu, J., Pinto, M. 1985. Epithelial properties of human colonic carcinoma cell line Caco-2: Effect of secretagogues. *Am. J. Physiol.* **248**:C410–C418
15. Grasset, E., Pinto, M., Dussaulx, E., Zweibaum, A., Desjeux, J.F. 1984. Epithelial properties of human colonic carcinoma cell line Caco-2: Electrical parameters. *Am. J. Physiol.* **247**:C260–C267
16. Hauri, H.P. 1986. Use of monoclonal antibodies to investigate the intracellular transport and biogenesis of intestinal brush-border proteins. *Biochem. Soc. Trans.* **14**:161–163
17. Hauri, H.P., Kedinger, M., Haffen, K., Grenier, J.F., Hadorn, B. 1975. Organ culture of human duodenum and jejunum. *Biol. Gastroenterol.* **8**:307–319
18. Hopfer, U., Nelson, K., Perrotto, J., Isselbacher, K.J. 1973. Glucose transport in isolated brush border membrane from rat small intestine. *J. Biol. Chem.* **248**:25–32
19. Kimmich, G.A., Randles, J. 1975. A Na^+-independent, phloretin-sensitive monosaccharide transport system in isolated intestinal epithelial cells. *J. Membrane Biol.* **23**:57–76
20. Kimmich, G.A., Randles, J. 1981. α-Methylglucoside satisfies only Na^+-dependent transport system of intestinal epithelium. *Am. J. Physiol.* **241**:C227–C232
21. Lee, A.J., McInerney, P.J., Mullins, P.R. 1984. Statcalc: An integrated statistics system for the Apple II microcomputer. *Comput. Prog. Biomed.* **18**:265–272
22. Lloyd, J.B., Whelan, W.J. 1969. An improved method for enzymic determination of glucose in the presence of maltose. *Anal. Biochem.* **30**:467–470
23. Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, R.J. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**:265–275
24. Maestracci, D., Preiser, H., Hedges, T., Schmitz, J., Crane, R.K. 1975. Enzymes of the human intestinal brush border membrane, identification after gel electrophoretic separation. *Biochim. Biophys. Acta* **383**:147–156
25. Maze, M., Gray, G.M. 1980. Intestinal brush border aminooligopeptidases: Cytosol precursors of the membrane enzyme. *Biochemistry* **19**:2351–2358
26. Mitchell, J.D., Mitchell, J., Peters, T.J. 1974. Enzyme changes in human small bowel during culture in vitro. *Gut* **15**:805–811
27. Mohrmann, I., Mohrmann, M., Biber, J., Murer, H. 1986. Sodium-dependent transport of Pi by an established intestinal epithelial cell line (Caco-2). *Am. J. Physiol.* **250**:G323–G330
28. Moog, F. 1981. Perinatal development of the enzymes of the brush border membrane. *In:* Textbook of Gastroenterology and Nutrition in Infancy. E. Lebenthal, editor. pp. 139–147. Raven, New York
29. Naftalin, L., Sexton, M., Whitaker, J.F., Randall, R.J. 1969. A routine procedure for estimating serum γ-glutamyltranspeptidase activity. *Clin. Chim. Acta* **26**:293–296
30. Noren, O., Sjostrom, H., Danielsen, E.M., Cowell, G.M., Skovbjerg, H. 1986. The enzymes of the enterocyte plasma membrane. *In:* Molecular and Cellular Basis of Digestion, P. Desnuelle, H. Sjostrom, and O. Noren, editors. pp 335–365. Elsevier Science Publishers, Amsterdam, New York, Oxford
31. Pinto, M., Robine-Leon, S., Appay, M.D., Kedinger, M., Triadou, N., Dussaulx, E., Lacroix, B., Simon-Assman, P., Haffen, K., Fogh, J., Zweibaum, A. 1983. Enterocyte-like differentiation and polarization of the human colon carcinoma cell line Caco-2 in culture. *Biol. Cell* **47**:323–330
32. Post, R.A., Sen, A.K. 1967. Sodium and potassium stimulated ATPase. *Meth. Enzymol.* **10**:762–768
33. Potter, G.D., Burlingame, S.M. 1986. Glucose-coupled sodium absorption in the developing rat colon. *Am. J. Physiol.* **250**:G221–G226
34. Puigserver, A., Wicker, C., Gaucher, C. 1986. Adaptation of pancreatic and intestinal hydrolases to dietary changes. *In:* Molecular and Cellular Basis of Digestion. P. Desnuelle, H. Sjostrom, and O. Noren, editors. pp. 113–124. Elsevier Science Publishers, Amsterdam, New York, Oxford
35. Ramond, M.J., Martinot-Peignoux, M., Erlinger, S. 1985. Dome formation in the human colon carcinoma cell line Caco-2 in culture. Influence of ouabain and permeable supports. *Biol. Cell.* **54**:89–92
36. Rousset, M. 1986. The human colon carcinoma cell lines HT-29 and Caco-2: Two in vitro models for the study of intestinal differentiation. *Biochimie* **68**:1035–1040
37. Rousset, M., Laburthe, M., Pinto, M., Chevalier, G., Rouyer-Fessard, C., Dussaulx, E., Trugnan, G., Boige, N., Brun, J.L., Zweibaum, A. 1985. Enterocytic differentiation and glucose utilization in the human colon tumor cell line Caco-2: Modulation by forskolin. *J. Cell. Physiol.* **123**:377–385
38. Schmitz, J., Preiser, H., Maestracci, D., Ghosh, B.K., Cerda, J.J., Crane, R.K. 1973. Purification of the human intestinal brush border membrane. *Biochim. Biophys. Acta* **323**:98–112
39. Seetharam, B., Yeh, K.Y., Moog, F., Alpers, D.H. 1977. Development of intestinal brush border membrane proteins in the rat. *Biochim. Biophys. Acta* **470**:424–436
40. Semenza, G., Kessler, M., Hosang, M., Weber, J., Schmidt, U. 1984. Biochemistry of the Na^+, D-glucose cotransporter of the small intestinal brush-border membrane. The state of the art in 1984. *Biochim. Biophys. Acta* **779**:343–379
41. Yamada, K., Moriuchi, S., Hosoya, N. 1979. Some characteristics of early appearing isomaltase in intestinal mucosa of suckling rat. *J. Nutr. Sci. Vitaminol.* **24**:177–184
42. Yokota, K., Nishi, Y., Takesue, Y. 1983. Effect of phloretin on Na-dependent D-glucose uptake by intestinal brush border membrane vesicles. *Biochem. Pharmacol.* **32**:3453–3457
43. Zweibaum, A., Triadou, N., Kedinger, M., Augeron, C., Robine-Leon, S., Pinto, M., Rousset, M., Haffen, K. 1983. Sucrase-isomaltase: A marker of foetal and malignant epithelial cells of the human colon. *Int. J. Cancer* **32**:407–412

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