Characterization of the D-glucose/Na+ cotransport system in the intestinal brush-border membrane by using the specific substrate, methyl α-D-glucopyranoside

Edith Brot-Laroche, Stéphane Supplisson, Brigitte Delhomme, Ana Isabel Alcalde and Francisco Alvarado

Centre de Recherches sur la Nutrition, Centre National de la Recherche Scientifique, Meudon (France)

By using isolated membrane vesicles, we have investigated the tenet that D-glucose transport across the intestinal brush-border membrane involves at least two distinct, Na+-activated agencies (D-glucose transport systems S-1 and S-2), only one of which (S-1) can use methyl α-D-glucopyranoside (methyl α-glucoside) as a substrate. Our results with this glucose analogue show that: (a) As a function of time, methyl α-glucoside uptake exhibits a typical overshoot, similar to but smaller than that given by D-glucose with the same vesicle batch. (b) Nonlinear regression analysis of substrate-saturation curves reveals that, contrary to D-glucose, methyl α-glucoside transport involves a single transport system which we have identified as S-1. (c) Methyl α-glucoside exhibits an apparent affinity (defined as the reciprocal of K_m) 4-times smaller than that of D-glucose for S-1 ($K_{m(Dglucose)} = 0.5$ mM; $K_{m(methyl\ \alpha\text{-glucoside})} = 2$ mM). However, methyl α-glucoside has a V_{max} (230 pmol/mg protein per s) identical to that characterizing D-glucose transport by this system. (d) In the absence of Na+, methyl α-glucoside uptake is indistinguishable from simple diffusion, confirming that Na+ is an obligatory activator of S-1. (e) Phlorizin behaves as a fully competitive inhibitor of methyl α-glucoside transport ($K_i = 18$ μM), again indicating that S-1 is involved. (f) Neither phloretin nor cytochalasin B affects methyl α-glucoside uptake. We conclude that methyl α-glucoside is a substrate specific for S-1, which permits study of the properties of this system without interference by substrate fluxes taking place through any other channel.

Introduction

The glucose analogue, methyl α-D-glucopyranoside (methyl α-glucoside) is widely used as a model substrate for the sugar/Na+ cotransport system of intestinal [1–4] and renal [5–13] brush-border membranes. In addition to being non-metabolizable, it has the advantage of not being transported by the sodium-independent glucose transport system of the basolateral membrane, which makes it specially suitable for studies with both intact tissue and isolated enterocytes [1,2]. With this last preparation in particular, methyl α-glucoside has been used to measure those fluxes taking place through the apical border without interference by possible losses across the basolateral membrane [2].

However, the work just quoted assumes that methyl α-glucoside utilizes a single transport system, an assumption now requiring validation in

Correspondence: Dr. F. Alvarado, Centre de Recherches sur la Nutrition du C.N.R.S., 9 rue Hetzel, 92190 Meudon, France.

view of recent evidence indicating that D-glucose transport across the intestinal brush-border membrane is mediated by at least two different Na^+-activated carriers [14,15]. The first, System 1 (S-1), involves a high-affinity, low-capacity carrier, the apparent affinity of which is unaffected by a temperature change from 25°C to 35°C. We have identified S-1 with the 'classical' intestinal D-glucose/Na^+ cotransport system which is known to be specific for, among other sugars, D-glucose, D-galactose, methyl α-glucoside and methyl β-glucoside [16]. The second, System 2 (S-2), is a low-affinity, high-capacity system exhibiting a dramatic change in kinetic behaviour as the temperature is increased from 25°C to 35°C. In effect, whereas S-2 exhibits diffusion-like kinetics at 25°C, at 35°C it is saturable, albeit at glucose concentrations much higher than those needed to saturate S-1. Although S-2 accepts D-glucose, D-galactose and methyl β-glucoside as substrates (or inhibitors), it does not recognize methyl α-glucoside as a competitive inhibitor [14].

If, as suggested by this evidence, methyl α-glucoside is a substrate specific only for S-1, an analysis of methyl α-glucoside uptake by isolated brush-border membrane vesicles should afford an opportunity of establishing unequivocally the properties of S-1, provided of course that the assumption is confirmed that, in contrast with D-glucose, methyl α-glucoside is transported only through this pathway. The necessary demonstration, which to the best of our knowledge has never been done before, is provided in this paper.

Preliminary accounts of this work have been given [17,18].

Materials and Methods

Animals. Adult guinea pigs of either sex were used. The animals (400–500 g) had free access to food and their drinking water contained sufficient vitamin C to maintain them in good health. After killing the animals by a blow on the neck, the jejunum was rapidly everted, washed with saline and stored at −20°C in sealed plastic bags. For one experiment, the frozen jejunum of one hog weighing 100 kg (Large White, Jouy-en-Josas) was used.

Reagents. All reagents were of analytical grade.

Sugar purity was checked by thin-layer chromatography with silica-gel-60 precoated aluminium sheets (0.2 mm thick, activated for 30 min at 100°C; Merck) with *n*-butanol/acetone/water (4/5/1, v/v) as the solvent. Cold methyl α-glucoside was freed from contaminating D-glucose by chromatography on Dowex-1 resin (2% cross linkage, stock 1 × 2–400 dry mesh 200–400) in the hydroxy form [19]. α-Methyl[U-^{14}C]glucoside (New England Nuclear Corporation) was purified by chromatography on Sephadex G-10 (Pharmacia Fine Chemicals, Uppsala, Sweden).

Brush-border membrane vesicle preparation. Vesicles were prepared by using the Mg^{2+}-EGTA precipitation method of Hauser et al. [20] with minor modifications. The final preparation contained 500 mM sorbitol and a metal-free, Hepes/maleic acid/*n*-butylamine buffer consisting of 10 mM Hepes and 7 mM *n*-butylamine, adjusted with maleic acid to pH 7.4 [21]. Protein was measured with the Bio-Rad Protein Assay Kit with serum γ-globulin as the standard. The vesicles, adjusted to 20–25 mg protein per ml, were stored in 200 μl batches in liquid nitrogen until the day of each transport experiment.

Assays. Substrate uptake was determined by using a rapid filtration technique, essentially as described by Hopfer et al. [22]. The vesicles were equilibrated with the Hepes/maleic acid/*n*-butylamine buffer (pH 7.4) and enough sorbitol to complete to 500 mosmol/l [14]. Unless stated otherwise, transport measurements were performed in a medium containing: (i) the Hepes/maleic acid/*n*-butylamine buffer (pH 7.4); (ii) the (U-^{14}C)-labeled substrate; (iii) either NaCl or KCl to give an initial metal gradient ([out/in] = 100/0 mM); and (iv) enough sorbitol to maintain an inward-directed net osmolarity gradient (osmolarity ratio [out/in] = 600/500 mosmol/l). Initial uptake rate measurements (0.5 to a few seconds) were performed at either 25°C or 35°C with a Short-Time Incubation Apparatus (Innovativ Labor, Switzerland [23]), as described [14].

Calculation and expression of results. Results are expressed as either absolute uptakes (units: picomol/mg protein), absolute velocities (picomol/mg protein per s), or as relative velocities (nl/mg protein per s), where the absolute velocities are divided by the substrate concentration [21].

Fit of the transport results to the Michaelis-Menten equation. Unless stated otherwise, uncorrected absolute velocities as a function of the substrate concentration were fitted by iteration to an equation containing two saturable, Michaelian terms, plus a diffusional component [24,25]:

$$v = \frac{V_1 \cdot [S]}{K_{m1} + [S]} + \frac{V_2 \cdot [S]}{K_{m2} + [S]} + K_{ds}[S] \tag{1}$$

where the suffixes 1 and 2 identify two distinct transport systems. When only one such system was present ($V_2 = 0$), as indicated by the lack of fit of the data to Eqn. 1, the simplified equation holds:

$$v = \frac{V \cdot [S]}{K_m + [S]} + K_{ds}[S] \tag{2}$$

Finally, when an inhibitor, I, was under investigation, the Michaelian term in Eqn. 2 was made to contain two additional parameters, Q and R, necessary to diagnose the type of inhibition. (We apply here the random allosteric model of Alvarado et al. [26] involving four dissociation constants related by the R parameter ($R = K_s/K_s' = K_a/K_a'$) and two rate coefficients related by Q ($Q = k'/k$).) The resulting equation remains Michaelian but the two key kinetic parameters, V and K_m, are hyperbolic functions of [I]:

$$v = \frac{[V(1 + QRz)/(1 + Rz)] \cdot [S]}{[K_s(1 + z)/(1 + Rz)] + [S]} + K_{ds}[S] \tag{3}$$

where $z = [I]/K_i$; $V_{(apparent)} = [V(1 + QRz)/(1 + Rz)]$, $K_m = [K_s(1 + z)/(1 + Rz)]$ and K_s is the dissociation constant of the substrate-carrier complex; see Ref. 26 for further details.

The kinetic diffusion constant, K_{ds}, was assessed by nonlinear regression analysis as the limiting slope of appropriate substrate-saturation curves, as described [14]. This and other details concerning the application of the above equations to our data are given in the text.

Statistical analyses. Individual uptake data points were compared by means of a one-way analysis of variance [27,28]. The nonlinear regression analyses included an *F*-test [25]. All calculations were performed by using an Apple MacIntosh microcomputer.

Results and Discussion

Time-course of D-glucose and methyl α-glucoside uptake by isolated brush-border membrane vesicles

At low concentrations (e.g., 0.1 mM), D-glucose uptake across the intestinal brush-border membrane occurs mainly through the high-affinity system, S-1, since S-2 is well below saturation [14]. We therefore began by comparing directly the time-course of uptake of glucose and methyl α-glucoside at this concentration. The results (Fig. 1) reveal that: (1) Both sugars exhibit identical relative uptakes at equilibrium, meaning that the vesicle volume available to them is the same (about 0.6 μl/mg protein); (2) the glucose initial entry rates are 5-times faster than those of methyl α-glucoside; (3) the hight of the glucose overshoot is 1.3-times that of methyl α-glucoside; (4) the two efflux curves are superimposable from 5 min onwards; and (5) glucose influx is linear only for the first 3–5 s, whereas that of methyl α-glucoside is linear for up to 30–40 s. We shall see below that all of these results can be explained simply on the basis of the lower affinity of methyl α-glucoside for S-1, as compared to that of glucose.

Substrate-saturation experiments: methyl α-glucoside transport involves a single transport system, S-1

When used as an inhibitor of D-glucose transport by isolated vesicles, methyl α-glucoside exhibited partial inhibition kinetics, which we have explained in terms of methyl α-glucoside being a substrate (hence, a fully competitive inhibitor) of the high-affinity S-1 but inert towards S-2 [14]. It was therefore of special interest to study the saturation kinetics of methyl α-glucoside transport to ascertain whether a single transport system is involved here, rather than the two participating in D-glucose transport. We measured initial methyl α-glucoside utpake rates (for either 2 or 10 s) by using a wide substrate concentration range, both at 25°C and at 35°C. Uncorrected uptake data were then analysed by nonlinear regression analysis as applied to Eqn. 1 which, the iterations showed, reduced to Eqn. 2 because V_2 was indistinguishable from zero (Table I). Taken together, these results show that methyl α-glucoside transport involves a single, high-affinity transport system, whose K_m is not affected by a temperature
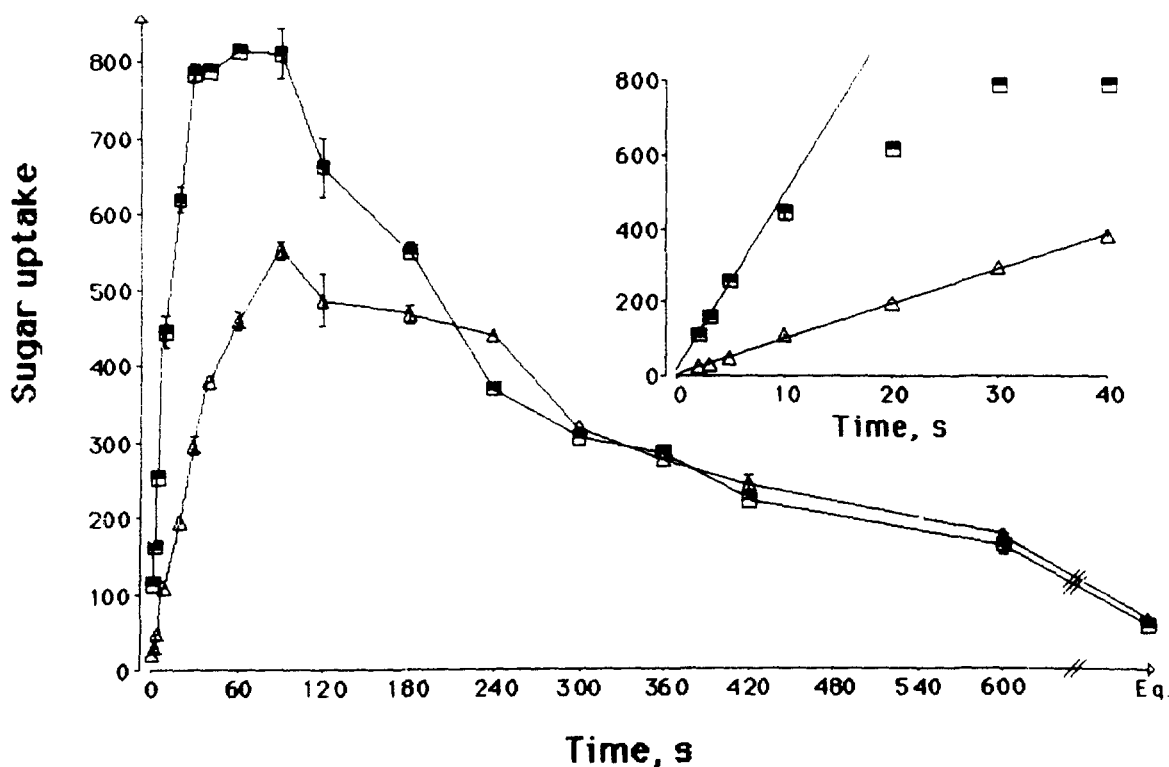
Fig. 1. Time-course of D-glucose and methyl α-glucoside transport by guinea-pig jejunal brush-border membrane vesicles. Uptakes were measured at room temperature under standard conditions which included: (i) an inward-directed NaCl gradient ($[Na]_{out}/[Na]_{in} = 100/0$ mM) and (ii) 0.11 mM of either methyl α-[U-^{14}C]glucoside (△) or D-[U-^{14}C]glucose (■) as substrate. Results are expressed as absolute uptakes (pmol/mg protein), each time point being the mean of three determinations ± S.E. (for most points, S.E. is smaller than the symbol). The equilibria were measured at 90 min. Inset: expanded representation of the initial 40 s.

change from 25 to 35°C, whereas its apparent V_{max} increases 3.7-times.

The K_m obtained for methyl α-glucoside transport in our experiments with isolated brush-border membrane vesicles is virtually identical to those obtained (when similar external NaCl concentra-

tions were used) either with intact tissue preparations [1,3], with isolated chicken enterocytes [29] or with isolated brush-border membrane vesicles from dog kidney cortex [9]. Furthermore, when the same biological material was used (guinea-pig jejunum), it is noteworthy that identical results

TABLE I

EFFECT OF TEMPERATURE ON THE KINETICS OF METHYL α-GLUCOSIDE TRANSPORT BY GUINEA-PIG JEJUNAL BRUSH-BORDER MEMBRANE VESICLES IN THE PRESENCE OF SODIUM

Uncorrected uptake rates were fitted to Eqn. 1 by nonlinear regression analysis. Figures in parentheses mean that K_{ds} was fixed to the values shown before attempting the fit. For the F-test (F[df]), the degrees of freedom for lack of fit and for pure error, respectively, are given between brackets [24].

Time (s)	Assay (°C)	V (pmol·mg^{-1}·s^{-1})	K_m (mM)	K_{ds} (nl·mg^{-1}·s^{-1})	F[df]
2	25	133 ± 29	1.9 ± 0.3	(5)	0.38 [9, 51]
2	35	490 ± 42	2.0 ± 0.3	(5)	1.20 [11, 65]
10	25	151 ± 17	2.2 ± 0.4	4.9 ± 0.9	0.73 [2, 10]

were obtained either with isolated vesicles (K_m about 2 mM, this paper) or with the tissue accumulation method ($K_m = 2.1$ mM [1]). This perfect agreement upholds our contention [30] that appropriate shaking can eliminate the interference caused by unstirred water layers on the kinetics of transport with intact tissue preparations, in vitro. The observations just discussed indicate strongly that, although the various preparations used represent quite different levels of functional complexity (see Ref. 18), all of them provide the means of measuring the same molecular event, the interaction of the (S-1) brush-border membrane carrier with the extracellular (or extravesicular) substrate.

To compensate for the variability often accompanying experiments with isolated vesicles, the kinetic parameters for either D-glucose or methyl α-glucoside transport were compared directly by using a single batch of brush-border membrane vesicles derived from a homogeneous group of guinea pigs. The results strongly support the tenet that methyl α-glucoside is a substrate only for S-1, exhibiting an apparent V_{max} identical to that of D-glucose for this system (V_1, Table II). At the same time, if the apparent affinity is defined as the reciprocal of K_m, these results demonstrate that the affinity of D-glucose for S-1 is 3.7-times larger than that of methyl α-glucoside, again agreeing with previous results obtained with intact-tissue preparations [16]. The conclusion therefore seems warranted that binding to the carrier, not the subsequent translocation step, is the parameter affected by the attachment to glucose of a methyl group in the C1-alpha position. The slower entry rate and smaller overshoot given by

methyl α-glucoside (Fig. 1) can be explained fully in these simple terms.

Further evidence that methyl α-glucoside is transported only by S-1 was then sought by using phlorizin and cytochalasin B, substances purported to be, respectively, either a fully competitive inhibitor or inert towards this system.

Effect of phlorizin on methyl α-glucoside transport

With intact tissue intestinal preparations, phlorizin has been described as a fully competitive inhibitor of intestinal sugar transport [31]. However, the demonstration that this transport involves at least two different sodium-activated systems, both present in the brush-border membrane [14], raises the question of the exact mechanism of action of phlorizin on either system. For instance, with isolated guinea-pig brush-border membrane vesicles, we have verified that phlorizin inhibits D-glucose transport competitively, but this clear-cut result was obtained only with glucose concentrations below 1 mM [16]. Otherwise, with isolated vesicles the kinetic effect of phlorizin on glucose transport does not appear to be simple, and can give ambiguous results whose elucidation would require additional work (for further details on this question, see Ref. 16).

A detailed study of the kinetics of methyl α-glucoside inhibition by phlorizin seemed necessary to confirm the double tenet that methyl α-glucoside is a substrate only for S-1 and that this is the system inhibited fully competitively by phlorizin [31]. The question was investigated by measuring the initial uptake rate at variable concentrations of both the substrate and the inhibitor.

TABLE II

KINETIC PARAMETERS OF D-GLUCOSE AND METHYL α-GLUCOSIDE TRANSPORT IN THE PRESENCE OF SODIUM, ESTIMATED BY USING THE SAME BRUSH-BORDER MEMBRANE VESICLE PREPARATION FROM GUINEA-PIG JEJUNUM

Saturation curves were constructed at 35°C under standard conditions which included an inward–directed NaCl gradient ([Na]$_{out}$/[Na]$_{in}$ = 100/0 mM) and either D-glucose (incubations of 3 s) or methyl α-glucoside (10 s) as the substrate. Uncorrected uptake rates were fitted to Eqn. 1 by nonlinear regression analysis. Figures in parentheses mean that the relevant parameter was fixed to the value shown before attempting the fit (the methyl α-glucoside results would not fit an equation involving two transport components). Units: V = pmol/mg protein per s; K_m = mM; K_{ds} = nl/mg protein per s.

Substrate	V_1	K_{m1}	V_2	K_{m2}	K_{ds}
D-Glucose	230 ± 31	0.68 ± 0.14	2320 ± 245	69 ± 15	(5)
Methyl α-glucoside	237 ± 16	2.52 ± 0.30	(0)	–	5.13 ± 0.60

The function $v = f$([methyl α-glucoside], [phlorizin]) was then analyzed mathematically in a single computer run by applying to the uncorrected, total uptake data a global nonlinear regression analysis based on Eqn. 3. The best fits (illustrated graphically in Fig. 2) indicate that, of the two critical parameters tested (see Material and Methods), R is indistinguishable from zero, meaning that phlorizin, as expected, is a pure, fully competitive inhibitor of methyl α-glucoside transport. Furthermore, this experiment yielded $K_i = 18$ μM for phlorizin, which is the same value we obtained previously [16] with D-glucose as substrate, provided that the substrate concentration did not exceed 1 mM, as already mentioned.

Lack of effect of cytochalasin B on methyl α-glucoside transport

Cytochalasin B has been described as a highly specific inhibitor of the basolateral membrane D-glucose transport system [32], so that inhibition
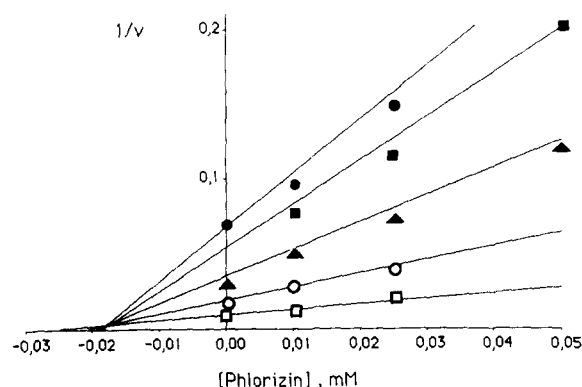

Fig. 2. Fully competitive effect of phlorizin on methyl α-glucoside transport. Uptakes were measured for 2 s at 35°C under standard conditions which included: (i) a NaCl gradient ([Na]$_{out}$/[Na]$_{in}$ = 100/0 mM); (ii) five different methyl α-glucoside concentrations (0.1 (●), 0.125 (■), 0.2 (▲), 0.4 (○), 1.0 (□) mM); and (iii) phlorizin concentrations ranging from 10 to 50 μM. The uncorrected uptake rates were fitted by nonlinear regression analysis as explained in the text. The net uptake rates (pmol/mg protein per s), corrected for diffusion (with a K_{ds} value of 4 nl/mg protein per s), are illustrated according to the $1/v = f$([I]) transformation. To show better the common point of crossing of all curves (indicative of fully competitive inhibition), not all of the results are illustrated. The straight lines shown are the theoretical ones computed by using the appropriate equation and the following (estimated) parameters: $V = 412 \pm 38$; $K_s = 2.74 \pm 0.33$ mM; $K_{i(phlorizin)} = 0.018 \pm 0.002$ mM and $R = 0$. The F-test yielded $F = 1.47$ [17,97].

by this agent is usually taken as proof of the presence of basolateral membrane vesicles in a given (e.g., brush-border membrane) preparation [32,33]. However, this simple interpretation requires reinvestigation in view of our own observation that, at high substrate concentrations, D-glucose uptake is inhibited partially by cytochalasin B [14]. This fact we have interpreted as due to the existence in the brush-border membrane of a second transport system (S-2), distinct from the one in the basolateral membrane but similarly sensitive to inhibition by cytochalasin B [14,18].

The results collected in Table III demonstrate the different effect of cytochalasin B on the transport of either D-glucose or methyl α-glucoside by isolated intestinal brush border membrane vesicles. As concerns D-glucose uptake, cytochalasin B is either inert or inhibits partially (38%) at 0.1 and 10 mM glucose concentrations, respectively. This result agrees fully with the double premise [14] that: (i) essentially all of the transport taking place at low glucose concentrations (e.g., 0.1 mM) occurs through the high-affinity system, S-1, which is insensitive to cytochalasin B inhibition and (ii) at 10 mM glucose, from 30 to 50% of the total uptake occurs through a second system, S-2, sensitive to cytochalasin B inhibition.

In contrast, cytochalasin B is inert towards methyl α-glucoside transport at both low and high substrate concentrations (Table III), confirming that this analogue is transported through a single channel, the cytochalasin-B-insensitive S-1.

The preceding conclusions are based on the assumption that our brush-border membrane preparations are not contaminated to any significant extent by basolateral membrane vesicles. Evidence to this effect has been presented before [14] and is upheld by new data shown in Table IV. Qualitatively, these results reveal that, with 10 mM D-glucose, total substrate uptake is inhibited by cytochalasin B, but not by either phloretin or theophylline, two classical inhibitors of the basolateral membrane carrier [32–36].

Furthermore, the data in Table IV permit the following quantitative calculations. First, from kinetic data obtained from the same lot of vesicles, total D-glucose uptake under the conditions of this experiment can be decomposed into transport due

TABLE III

EFFECT OF CYTOCHALASIN B ON THE UPTAKE OF EITHER D-GLUCOSE OR METHYL α-GLUCOSIDE IN THE PRESENCE OF SODIUM

The uptake of either D-glucose or methyl α-glucoside by guinea-pig jejunal brush-border membrane vesicles was measured at 35 °C under standard conditions which included the substrate and cytochalasin B concentrations shown, in the presence of an inward-directed NaCl gradient ($[Na]_{out}/[Na]_{in} = 100/0$ mM). All incubation media, including the controls, contained 0.5% ethanol, necessary to dissolve the cytochalasin B. Results are expressed as absolute uptakes in pmol/mg protein $\pm$ S.E. (n). Identical lower-case letters in parentheses identify results found to be statistically indistinguishable according to a one-way analysis of variance.

[S] (mM)	[Cytochalasin B] (mM)	Uptake (pmol/mg protein)	
		D-glucose	methyl α-glucoside
0.1	0	690 ± 28 (6)(a)	122 ± 6 (3)(b)
0.1	0.1	670 ± 20 (6)(a)	122 ± 5 (5)(b)
10	0	6 660 ± 169 (6)(c)	2 478 ± 32 (5)(e)
10	0.1	4 150 ± 282 (6)(d)	2 304 ± 117 (5)(e)

to either S-1 (62%) or S-2 (31%), plus a diffusion component (7%) which is virtually identical to that determined directly with L-glucose (9%). Second, phloretin and theophylline are totally without effect, meaning that both are inert towards S-1 and towards S-2. Third, 0.2 mM cytochalasin B inhibits partially, a result which, together with the data in Table III, indicates that, while cytochalasin B is inert towards S-1, it inhibits S-2 essentially completely (compare the 25% inhibition caused by cytochalasin B and the 31% calculated to correspond to S-2; it is doubtful that the missing 6% has any statistically significant meaning). Finally, the data suggest another interesting conclusion. If 0.7 mM phlorizin can be expected to inhibit S-1 fully, the partial inhibition observed in Table IV (57%) suggests that S-2 (which amounts to 62% of the total uptake) is not affected by this inhibitor.

Na⁺ is an obligatory activator of methyl α-glucoside uptake

Previous work with glucose as the substrate showed that, although Na^+ is by far the strongest activator among the alkali-metal ions, stereospecific D-glucose transport occurs nonetheless in the absence of this ion.

Saturable D-glucose uptake was found in the

TABLE IV

EFFECT OF TRANSPORT INHIBITORS ON D-GLUCOSE UPTAKE BY GUINEA-PIG BRUSH-BORDER MEMBRANE VESICLES IN THE PRESENCE OF SODIUM

The uptake of 10 mM of either D-glucose or L-glucose was measured for 3 s at 35 °C in the presence of an inward-directed NaCl gradient ($[Na]_{out}/[Na]_{in} = 100/0$ mM) and the indicated effectors. The results (pmol/mg protein per s $\pm$ S.E. (n)) are the means of 7–9 determinations made with two different brush-border membrane preparations. Identical lower-case letters in parentheses identify results found to be statistically indistinguishable according to a one-way analysis of variance.

Substrate	Effector	Uptake	% control
D-Glucose	none	2 290 ± 75 (8)(a)	100
	phloretin (0.25 mM)	2 642 ± 94 (9)(a)	115
	phlorizin (0.7 mM)	888 ± 28 (9)(b)	39
	theophillin (5 mM)	2 279 ± 54 (9)(a)	99
	cytochalasin B (0.2 mM)	1 590 ± 74 (7)(c)	69
L-Glucose	none	437 ± 13 (7)(d)	11

presence of either Li$^+$ (K_m = 25 mM) or K$^+$ (K_m = 60 mM), this last being indistinguishable from that obtained in the absence of any metal, i.e., when all metal ions were substituted by an iso-osmotic amount of the nonelectrolyte, sorbitol [14]. These results could be interpreted in either of two ways. Firstly, Na$^+$ could be a nonessential activator of a single cotransport system of low cationic specificity. Secondly, there could be two or more cation-activated transport systems in the brush border, only one of them being the one we have postulated [37] to be obligatorily activated by Na$^+$. The second of these two hypotheses was upheld by the demonstration that, in our brush-border membrane preparations, there are at least two distinct sodium-activated D-glucose transport systems, only one of which (S-1) is sensitive to methyl α-glucoside inhibition [14].

The difference between D-glucose and methyl α-glucoside is demonstrated further by the experiment in Table V. These results reveal that, with the alkali-metal ion electro-chemical gradient used ([cation]$_{out}$/[cation]$_{in}$ = 100/0 mM), Na$^+$ indeed behaves as an obligatory activator of S-1, since the relative uptake of methyl α-glucoside drops to levels indistinguishable from that of the diffusion marker, L-glucose, under any condition involving absence of Na$^+$.

Because of the restricted methyl α-glucoside concentration range used in the preceding experiment (0.1 and 10 mM), we investigated in greater
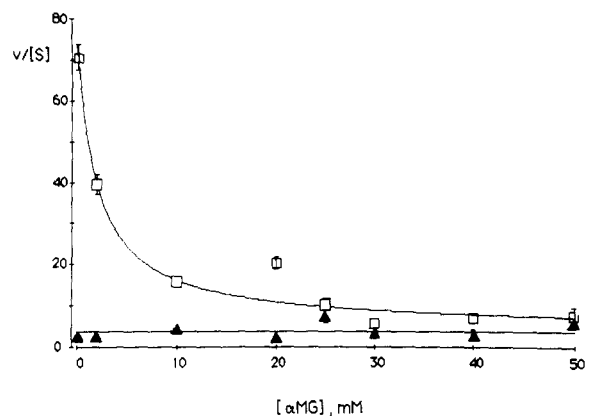

Fig. 3. Transport of methyl α-glucoside as a function of concentration, in the presence and in the absence of Na$^+$. Uptake was measured for 10 s at 23°C under standard conditions which included: (i) methyl α-glucoside (αMG) concentrations ranging from 0.1 to 50 mM and (ii) an inward-directed gradient ([out]/[in] = 100/0 mM) of the chloride salt of either Na$^+$ ($\square$) or K$^+$ ($\blacktriangle$). The results are expressed as relative velocities (nl/mg protein per s ± S.E.; n = 3 to 6). The methyl α-glucoside relative uptake rates in the presence of K$^+$ are all statistically homogeneous according to a one-way analysis of variance. Therefore, their average value defines the diffusion boundary (3.7 ± 0.4 nl/mg protein per s), illustrated by the horizontal line. The uptake curve in the presence of Na$^+$ converges with this line at methyl α-glucoside concentrations above 30 mM.

detail the relative effecs of Na$^+$ and of K$^+$, using a larger range of substrate concentrations (0.1 to 50 mM). The results (Fig. 3) confirm the obliga-

TABLE V

EFFECT OF THE ALKALI-METAL IONS ON METHYL α-GLUCOSIDE UPTAKE

The uptake of either methyl α-glucoside or the diffusion marker L-glucose was measured for 10 s at 25°C under standard conditions which included: (i) ^{14}C-labeled sugar at the indicated concentrations; and (ii) either an inward-directed gradient ([out]/[in] = 100/0 mM) of the chloride salt of the indicated metals or 200 mM sorbitol ('none'). The results, expressed as relative uptake rates (nl/mg protein per s ± S.E. (n)) are the means of 3–8 separate determinations. In the absence of Na$^+$, the methyl α-glucoside relative uptake rates are all concentration-independent and statistically indistinguishable from the L-glucose results. Therefore, all of these data (labeled 'a') were pooled to calculate the relative uptake rate due to simple diffusion: 3.7 ± 0.3 nl/mg protein per s (n = 44).

Substrate	Metal	Substrate concn. (mM):	Relative uptake rate (nl·mg^{-1} protein·s^{-1})		
			0.1	10	40
Methyl α-glucoside	Na$^+$		82.7 ± 0.5 (8)	14.3 ± 0.8 (6)	
	Li$^+$		3.5 ± 0.8 (4)(a)	3.0 ± 0.9 (8)(a)	
	K$^+$		3.5 ± 1.1 (6)(a)	3.9 ± 0.8 (2)(a)	3.4 ± 0.9 (3)(a)
	none		2.7 ± 0.9 (4)(a)	3.4 ± 0.5 (8)(a)	
L-Glucose	Na$^+$		3.5 ± 0.9 (4)(a)	4.7 ± 1.4 (4)(a)	

tory nature of Na^+ activation because (i) in the presence of Na^+, the methyl α-glucoside relative uptakes are a hyperbolic function of the substrate concentration, indicating saturable transport, whereas (ii) when Na^+ is substituted by K^+, the methyl α-glucoside relative uptakes are constant and independent of substrate concentration, indicating uptake by simple diffusion. Quantitatively, the agreement between these results and others presented before [5] and in this paper is excellent. The upper curve in Fig. 3 represents a theoretical fit to a Michaelian hyperbola, computed by applying the kinetic parameters of methyl α-glucoside transport estimated in the experiments listed in Table I. The fit seems quite good in spite of the fact that the experiments in Table I involved two different lots of membrane vesicles. The diffusion constant calculated from the K^+ data (3.7 nl/mg protein per s: see Fig. 3) is identical to that obtained in the experiment of Table V, as well as with that found previously, either by using L-glucose or D-mannitol as diffusion markers, or by estimating the limiting slope of D-glucose uptake saturation curves by nonlinear regression analysis [14].

Effect of membrane potential on D-glucose and methyl α-glucoside transport by S-1

The D-glucose/Na^+ cotransporter of the intestinal brush border membrane is known to be electrogenic [38], as a result of which the membrane potential ($\Delta\psi$) affects the uptake of both D-glucose and Na^+. Use of methyl α-glucoside as

the substrate offered now the possibility of investigating the effect of membrane potential on S-1 without interference by any uptake possibly taking place through S-2.

In a first attempt at solving this question, we carried out methyl α-glucoside saturation experiments in the presence of a constant sodium gradient and two different membrane potentials, about zero and -59 mV. Our results with guinea-pig vesicles indeed indicate that $\Delta\psi$ affects exclusively $V_{\max}$ (see Table VI).

An equivalent experiment was carried out with pig brush-border membrane vesicles and D-glucose as the substrate, with qualitatively identical results. This particular experiment was performed at $22°C$, conditions under which S-2 is not activated (Supplisson, S., unpublished results), thereby behaving in practice as a diffusion [14]. This absence of saturability explains the high K_{ds} obtained with the pig vesicles, about 10-times larger than those observed for the guinea pig (Table VI). This means that, with pig vesicles at this temperature, S-1 can be studied without interference, even when D-glucose is the substrate. The results show that, as was the case with the guinea pig, increasing the negativity of the membrane potential from about zero to -59 mV caused a 3-fold increase in $V_{\max}$ while K_m remained unaffected.

Both of the experiments in Table VI were conducted at constant Na^+ concentrations, so that the affinity-type activating effect of this ion can be considered to be invariant and, therefore, did not complicate the picture. This permitted the effect

TABLE VI

EFFECT OF MEMBRANE POTENTIAL ON THE KINETICS OF SUGAR/Na^+ COTRANSPORT

Saturation curves were performed at $25°C$ using either pig or guinea-pig jejunal brush-border membrane vesicles under standard conditions that included: (i) Radiolabeled substrate concentrations ranging from 0.1 to 50 mM; (ii) an inward-directed NaCl gradient ($[Na]_{out}/[Na]_{in} = 100/0$ mM); and (ii) vesicles preequilibrated with KCl to obtain upon dilution a zero-time K^+ gradient of $[K]_{out}/[K]_{in} = 10/100$ mM ($E_K = -59$ mV). During these preincubations, valinomycin (Val: 10 μg/mg protein) was either present (yes) or absent (no). Incubations were for either 2 s (D-glucose) or 10 s (methyl α-glucoside). Uncorrected initial uptake rates were fitted to Eqn. 1 by nonlinear regression analysis. Units: V = pmol/mg protein per s; K_m = mM; K_{ds} = nl/mg protein per s. See text for further details.

Substrate	Animal	Val	V	K_m	K_{ds}
Methyl α-glucoside	guinea pig	yes	262 ± 19	1.65 ± 0.19	2.1 ± 0.6
Methyl α-glucoside	guinea pig	no	119 ± 22	2.51 ± 0.64	1.4 ± 0.6
D-Glucose	pig	yes	462 ± 43	0.49 ± 0.11	29 ± 1.8
D-Glucose	pig	no	149 ± 19	0.47 ± 0.08	32 ± 2.8

of $\Delta \psi$ on the translocation step to manifest itself unambiguously.

Conclusion

The evidence collected in this paper confirms the existence of two distinct sodium-activated systems for D-glucose transport across the intestinal brush-border membrane. Methyl α-glucoside is a substrate for only one of these systems, S-1, which we have identified as being identical to the classical D-glucose/Na$^+$ cotransport system, defined in the early 60's (see Ref. 16). Use of methyl α-glucoside, therefore, permits study of the properties of S-1 without interference by substrate fluxes that otherwise can take place through other channels in the same membrane, as is the case with D-glucose.

Acknowledgments

References

1 Alvarado, F. (1976) in Intestinal Ion Transport (Robinson, J.W.L., ed.), 117–152, MTP Press, Lancaster

2 Kimmich, G.A. and Randles, J. (1981) Am. J. Physiol. 241, C227–C232

3 Robinson, J.W.L., Van Melle, G. and Johansen, S. (1983) in Intestinal Transport (Gilles-Baillien, M. and Gilles, R., eds.), pp. 64–75, Springer Verlag, Berlin

4 Boyd, C.A.R. and Parsons, D.S. (1979) J. Physiol. (London) 287, 371–391

5 Ullrich, K.J., Rumrich, G. and Klöss, S. (1974) Pflügers Arch. 351, 35–48

6 Rea, C. and Segal, S. (1973) Biochim. Biophys. Acta 311, 615–624

7 Kippen, I., Hirayama, B., Klinenberg, J.R. and Wright, E.M. (1979) Biochim. Biophys. Acta 558, 126–135

8 Rabito, C.A. (1981) Biochim. Biophys. Acta 649, 286–296

9 Turner, R.J. and Silverman, M. (1978) Biochim. Biophys. Acta 511, 470–486

10 Roth, K.S., Spencer, P.D., Higgins, E.S. and Spencer, R.F. (1985) Biochim. Biophys. Acta 820, 140–146

11 Reynolds, R. and Segal, S. (1974) Am. J. Physiol. 226, 791–795

12 Turner, R.J. and Silverman, M. (1977) Proc. Natl. Acad. Sci. (USA) 74, 2825–2829

13 Hsu, B.Y.L., Marshall, C.M., Corcoran, S.M. and Segal, S. (1982) Biochim. Biophys. Acta 692, 41–51

14 Brot-Laroche, E., Serrano, M.A., Delhomme, B. and Alvarado, F. (1986) J. Biol. Chem. 261, 6168–6176

15 Keljo, D.J., MacLeod, R.J., Perdue, M.H., Butler, D.G. and Hamilton, J.R. (1985) Am. J. Physiol. 249, G751–G760

16 Brot-Laroche, E. and Alvarado, F. (1983) in Intestinal Transport (Gilles-Baillien, M. and Gilles, R., eds.), pp. 147–169, Springer-Verlag, Berlin

17 Brot-Laroche, E., Serrano, M.A., Delhomme, B. and Alvarado, F .(1986) in Ion Gradient-Coupled Transport (Alvarado, F. and Van Os, C., eds.), pp. 137–140, Elsevier, Amsterdam

18 Alvarado, F., Brot-Laroche, E., Delhomme, B., Serrano, M.A. and Supplisson, S. (1986) Bolletino della Società Italiana di Biologia Sperimentale 62, 110–131

19 Austin, P.W., Hardy, F.E., Buchanan, J.G. and Baddiley, J. (1963) J. Chem. Soc. 1021, 5350–5353

20 Hauser, H., Howell, K., Dawson, R.M.C. and Bowyer, D.E. (1980) Biochim. Biophys. Acta 602, 567–577

21 Brot-Laroche, E. and Alvarado, F. (1984) Biochim. Biophys. Acta 775, 175–181

22 Hopfer, U., Nelson, K., Perroto, J. and Isselbacher, K.J. (1973) J. Biol. Chem. 248, 25–32

23 Kessler, M., Tannenbaum, V. and Tannenbaum, C. (1978) Biochim. Biophys. Acta 509, 348–359

24 Van Melle, G. and Robinson, J.W.L. (1981) J. Physiol. (Paris) 77, 1011–1016

25 Robinson, J.W.L. and Van Melle, G. (1983) J. Physiol. (London) 344, 177–187

26 Alvarado, F., Mahmood, A., Tellier, C. and Vasseur, M. (1980) Biochim. Biophys. Acta 613, 140–152

27 Snedecor, G.W. and Cochran, W.G. (1967) Statistical Methods, 6th Edn., Ch. 11, Iowa State University Press, Ames, IA

28 Dixon, W.J. and Massey, F.J., Jr (1969) in Introduction to Statistical Analysis, Ch. 10, MacGraw-Hill, New York

29 Kimmich, G.A. and Randles, J. (1984) Am. J. Physiol. 247, C74–C82

30 Lherminier, M. and Alvarado, F. (1981) Pflügers Arch. 389, 155–158

31 Alvarado, F. and Crane, R.K. (1962) Biochim. Biophys. Acta 56, 170–172

32 Kinne, R., Murer, H., Kinne-Saffran, E., Thees, M. and Sachs, G. (1975) J. Membrane Biol. 21, 375–395

33 Kinne, R. (1976) Curr. Topics Membrane Transp. 8, 209–267

34 Wright, E.M., Van Os, C.H. and Mircheff, A.K. (1980) Biochim. Biophys. Acta 597, 112–124

35 Kimmich, G.A. and Randles, J. (1978) Membrane Biochem. 1, 221–237

36 Kimmich, G.A. and Randles, J. (1975) J. Membrane Biol. 23, 57–76

37 Alvarado, F. and Lherminier, M. (1982) J. Physiol. (Paris) 78, 131–145

38 Murer, H. and Hopfer, U. (1974) Proc. Natl. Acad. Sci. USA 71, 484–488