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Analysis of Kinetic Data in Transport Studies: New Insights from Kinetic Studies of Na^+-D-Glucose Cotransport in Human Intestinal Brush-Border Membrane Vesicles Using a Fast Sampling, Rapid Filtration Apparatus

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Summary. Using the fast sampling, rapid filtration apparatus (FSRFA) recently developed in our laboratory (Berteloot et al., 1991. *J. Membrane Biol.* **122**:111–125), we have studied the kinetic characteristics of Na^+-D-glucose cotransport in brush-border membrane vesicles isolated from normal adult human jejunum. True initial rates of transport have been determined at both 20 and 35°C using a dynamic approach which involves linear-regression analysis over nine time points equally spaced over 4.5 or 2.7 sec, respectively. When the tracer rate of transport was studied as a function of unlabeled substrate concentrations added to the incubation medium, a displacement curve was generated which can be analyzed by nonlinear regression using equations which take into account the competitive inhibition of tracer flux by unlabeled substrate. This approach was made imperative since at 20°C, in the presence of high substrate concentrations or 1 mM phlorizin, no measurable diffusion was found and the resultant zero slope values cannot be expressed into a classical *v versus S* plot. All together, our results support the existence of a single Na^+-D-glucose cotransport system in these membranes for which Na^+ is mandatory for uptake. This conclusion is at variance with that of a recent report using the same preparation (Harig et al., 1989. *Am J. Physiol.* **256**:8618–8623). Since the discrepancy seems difficult to resolve on the consideration of experimental conditions alone, we have determined the kinetic parameters of D-glucose transport using one time point measurements and linear transformations of the Michaelis-Menten equation, in order to investigate the potential problems of such a widely used procedure. Comparing these approaches, we conclude that: (*i*) the dynamic uptake measurements give a better understanding of the different uptake components involved; (*ii*) it does not matter whether a dynamic or a one time point approach is chosen to generate the uptake data provided that a nonlinear-regression analysis with proper weighting of the data points is performed; (*iii*) analytical procedures which rely on linearization of Michaelian process(es) are endowed with a number of difficulties which make them unsuitable to resolve multicomponent systems in transport studies. A more general procedure which uses a nonlinear-regression analysis and a displacement curve is proposed since we demonstrate that it is far superior in terms of rapidity, data interpretation, and visual information.

Key Words Na^+-D-glucose cotransport · brush-border membrane vesicles · adult human jejunum · kinetic data · nonlinear-regression analysis

I. Introduction

Within the last few years, our laboratory has been very interested in the ontogeny of Na^+-dependent D-glucose transport function in the human small intestine [25–27]. While our early studies were successful in demonstrating the presence of this transport activity in brush-border membrane vesicles isolated from the jejunum and ileum of the 17- to 20-week-old human fetus [27], our later studies concluded that the functional heterogeneity in this transport capacity along the human small intestine underwent an early differentiation [25, 26]. Actually, both high-affinity, low-capacity and low-affinity, high-capacity pathways could be separated according to different kinetic criteria including kinetic parameter determination [25], Na^+ stoichiometry [25], sensitivity to inhibitors [26], and substrate specificity [26]. These results thus raised the question as to whether these two systems actually correspond to the transport systems in the adult or represent transitory forms to be found only during the fetal life. Recently, Harig et al. [13] provided kinetic evidence for the presence of two Na^+-D-glucose cotransport systems in the adult human jejunum, which could be viewed as supporting the continuation of the fetal forms. However, this study does not exclude the possibility of differentiation, and we decided to characterize further the kinetics of Na^+-D-glucose cotransport in human jejunal brush-border membrane vesicles. In contrast to the results reported by Harig et al. [13], we failed to demonstrate more than one Na^+-dependent transport pathway in these preparations. Since our studies were done using the fast sampling, rapid filtration apparatus (FSRFA) described in the previous paper [5] and nonlinear regression analysis of the rate over substrate concentration plots, the question was thus raised as to why two different approaches

using the same preparation and very similar experimental conditions (0-mV voltage-clamped conditions) would lead to such different results.

Kinetic evidence for multiple D-glucose cotransport pathways in brush-border membrane vesicle studies has been reviewed recently [20, 33] and reported, just to cite a few, in the rabbit [8], bovine [18], guinea-pig [1] and rat [10] small intestines. In contrast, studies performed in the chicken enterocyte (reviewed in ref. [20]) have failed to reveal such a heterogeneity in transport function. Obviously, because of species and experimental differences, it may appear quite difficult to integrate all this information within a unifying and unique explanation. In this context, it is worth noting that in all of the brush-border membrane vesicle studies cited above but one [8], the initial rates of transport have been estimated from replicates of a one time point measurement, generally at 3 sec from initiation of vesicle incubation with the substrate. Also, in all of these studies but one [1], the initial rate data have been analyzed according to the Eadie-Hofstee transformation and, in all cases, curvilinear plots have been reported. Thus, one may question the influence of this approach on the determination of kinetic parameters in transport studies. In any case, such a question is particularly relevant to the human studies discussed above.

It is quite clear that all kinetic studies on sugar transport by intestinal brush-border membrane vesicles have led to the general concensus that D-glucose uptake occurs through at least one saturable component plus a nonspecific pathway likely to represent passive diffusion. Thus, as discussed by Berteloot and Semenza [6], the analysis and interpretation of such kinetic data are complicated by the fact that the usual linear transformations of the Michaelis-Menten equation are, indeed, not linear. It is thus current practice to estimate the nonspecific component in a separate experiment, generally in the absence of Na^+ or in the presence of a saturating concentration of phlorizin, and to subtract its contribution from the total uptake data in order to isolate the saturating component(s). These data are then analyzed by linear regression or iterative procedure from the still nonlinear Eadie-Hofstee transformation [11]. This transformation is actually preferred for its higher sensitivity to deviations from linearity [9] which thus serves as a diagnostic test in assessing for the presence of multiple transport pathways. What actually seems to be a rationale approach is however outweighed by the following considerations: (*i*) The linear transformations distort (usually magnify) the experimental variability, thus leading to biased estimates in the values of the parameters [11, 12, 31]. This is particularly obvious with the Eadie-Hofstee plot where the dependent variable "v" (initial rate), subject to experimental error, appears on both axis [31]. Moreover, such a situation actually violates one of the fundamental assumptions for using a linear-regression approach [24]. (*ii*) The statistical information as to the determination of the nonspecific component is lost during the subtraction procedure, and thus, during the computation of the kinetic parameters for the saturating components. (*iii*) The experimental conditions are not necessarily identical when estimating total uptake and diffusion separately, and it may be difficult to assess subtle changes in the nonspecific component under these conditions. Obviously, even small errors during the subtraction can distort the linearization process such as to induce upward deviations that will mimick an heterogeneity in binding sites. As already pioneered by others in the field of transport [30, 34], it thus appears that a more general and accurate approach to solve these problems would be to apply a nonlinear-regression analysis to such data since: (*i*) Model equations which include both the saturable and nonspecific components can be directly fitted to the total uptake curve. It thus becomes possible to critically evaluate, on a pure statistical basis, the best fit model to the data points. (*ii*) Both data corrections which automatically increase the experimental variation and equation transformations which distort the error structure in the experimental data are avoided [22, 30, 34]. (*iii*) Nonlinear regression is in general less sensitive to the spacing and number of data points than linear regression [12, 31].

As briefly mentioned in a previous paragraph, nearly all kinetic studies related to Na^+-dependent D-glucose transport in intestinal brush-border membrane vesicles have used a one time point approach for the initial rate determination of the uptake process. Obviously, the validity of this approach is straightforward provided that two basic conditions are validated: (*i*) the chosen time point is taken as short as possible such as to belong to the linear part of the uptake time curve, in order to comply with the steady-state assumption underlying the analysis of such kinetic data [33]; and (*ii*) this prerequesite is fulfilled over the whole range of concentrations and experimental conditions within the same experiment. While very easy to satisfy and control using a semiautomatic apparatus as described by Kessler, Tannenbaum and Tannenbaum [19] and used for example in [1], these conditions may, however, be more difficult to demonstrate with the manual filtration technique because of the poor time resolution that can be achieved with this set up. Such limitations have been well perceived by Dorando and Crane [8] who performed multiple uptake measure-

ments over a limited time range and estimated the initial transport rates by polynomial regression of the uptake time points, as seem to be accepted by enzymologists [29], although this approach also presents some problems [2–4], as discussed recently [6].

In the present paper, we thus report our kinetic parameter determinations of Na^+-dependent D-glucose transport in adult jejunal brush-border membrane vesicles using the FSRFA [5]. We next compare the dynamic approach allowed by this technique with the more classical one time point measurement. We also compare the outcome to be expected by using either linear- or nonlinear-regression analysis with these two procedures. We thus demonstrate that both approaches turn out to be quite comparable provided that nonlinear-regression analysis is used, but that misinterpretations as to the meaning of the nonspecific component can easily occur using the one time point approach. We conclude that a single Na^+-D-glucose cotransport system is present in the adult human jejunum at both 20 and 35°C, at least under our 0-mV voltage-clamped conditions. We finally propose an alternative graphical representation which better accounts for the reality of the transport data and provides a better visual assessment of the fitted parameters compared to the usual *v versus S* plot.

II. Materials and Methods

A. Chemicals

Labeled compounds, D-(1-^{3}H(*n*))glucose (15.5 Ci/mmol) and D-(U-^{14}C)glucose (315 mCi/mmol) were purchased from New England Nuclear, and D-(1-^{14}C)mannitol (59 mCi/mmol) was obtained from Amersham. Valinomycin was obtained from Sigma Chemical and phlorizin from Aldrich Chemical. Amiloride hydrochloride was a gift from Merck, Sharp and Dohme Canada, Kirkland, Québec. All salts and chemicals used for buffer preparation were of the highest purity available.

B. Preparation of Brush-Border Membrane Vesicles

Adult human jejunum was obtained from three healthy organ donors with the kind collaboration of Dr. Jean Cardinal from Maisonneuve-Rosemont Hospital and Dr. Jacques Corman from Notre-Dame Hospital. The tissues were rinsed with ice-cold saline solution and frozen at −70°C until further processing. Intestinal brush-border membranes were purified by the $CaCl_2$ precipitation method of Schmitz et al. [32], and vesicles were prepared as previously described [25]. The P_2 fractions were resuspended in 50 mM Tris-HEPES buffer (pH 7.5) containing 0.1 mM $MgSO_4$, 250 mM KCl and a cocktail of protease inhibitors (pepstatin 0.01 mg/ml, chymostatin 0.01 mg/ml, aprotinin 1.25 μg/ml, and bacitracin 0.1 mg/ml). These fractions were aliquoted and stored in liquid nitrogen until use. The final steps in the preparation of brush-border membrane vesicles were done on the day of the experiment after resuspension of thawed P_2 fractions in the medium required for the transport studies. The vesicles so prepared showed stable glucose transport activities for at least six weeks from freezing (*data not shown*). The purity of the vesicle preparations was routinely determined by assaying sucrase activity in both the starting homogenates and the vesicle fractions.

C. Transport Studies

Brush-border membranes vesicles were usually resuspended to a final concentration of 9–11 mg/ml in the final resuspension buffer, and aliquots of 20 μl/0.5 ml incubation medium were used. The exact composition of the resuspension buffers and incubation media are given in the legends to the figures. All experiments have been performed under 0 mV voltage-clamped conditions using K^+-valinomycin, and 0.5 mM amiloride was included in all uptake media as recently reported [25].

All transport experiments were done according to the rapid filtration technique of Hopfer et al. [17] using the FSRFA described in the preceding paper [5]. Briefly, this apparatus allows for sampling of up to 18 aliquots from the same incubation mixture and fully automates the sequence of vesicle injection and mixing with the incubation medium, samplings, washings, and filtrations [5]. For initial rate determinations of glucose uptake at 20 and 35°C, nine aliquots of 50 μl each were taken at 0.5- or 0.3-sec intervals, respectively. Each aliquot was injected into 1 ml ice-cold stop solution (composition adjusted to match the final concentrations of the different species in the incubation medium) containing 1 mM phlorizin, filtered on 0.65-μm (Micro Filtration System) nitrogen cellulose filters and washed twice with 1 ml nonradioactive ice-cold stop solution.

When comparing the dynamic and the one time point approaches, the same sequence as above was followed, but we also introduced three more samplings at 2.75, 3 and 3.25 sec (8% variation). These three uptake values were averaged as a 3-sec time point, generating a standard error on the order of 6–12% around the mean value. It should be noted that the SD of regression estimated from the uptake-time curves generated by the FSRFA is in the order of 10 to 25% for the whole range of substrate concentration studied. The 6–12% variation found at 3 sec is much smaller, and thus, cannot represent an overestimation of the error usually generated from triplicates obtained at one time point, especially by using the manual procedure.

Filters were dissolved in minivials by 15-min incubation with 5 ml Filter Count (United Technologies Packard) and continuous shaking. ^{3}H and ^{14}C radioactivities were determined using a Minaxi Tri-Carb Series 4000, model 4450 scintillation counter (United Technologies Packard). All vials were counted for 5 min.

D. Assays

Marker enzyme for the brush-border membrane, namely sucrase (EC 3.2.1.48), was routinely assayed by a continuous spectrophotometric assay as proposed by Kunst, Draeger and Ziegenhorn [21]. Results are expressed as μmol of substrate hydrolyzed · hr^{-1} · mg $protein^{-1}$. Proteins were assayed using the BCA Protein Assay Reagent (Pierce Chemical) and bovine serum albumin as standard.

Table 1. Purification of BBMVs from adult human jejunum

	Specimen #1		Specimen #2	
	Mucosa	BBMV	Mucosa	BBMV
Protein content (g)	3.78	0.037 (0.97%)[a]	3.97	0.034 (0.86%)
Sucrase activity (μmol sucrose hydrolyzed · h^{-1} · mg protein^{-1})	142.1	1251.2 (8.8)[b]	44.7	529.4 (11.8)

[a] Percentage of recovery.
[b] Enrichment factor.

E. Data Analysis

Transport data are expressed as pmol or nmol solute uptake · mg protein^{-1}. When using the FSRFA, the initial rates of transport were determined by linear-regression analysis of the uptake time courses (nine data points). All kinetic parameter determinations (values ± SD of regression) were done using nonlinear-regression analysis. The same software (Enzfitter, Robin J. Leatherbarrow, Copyright © 1987, Elsevier-Biosoft) was used for both purposes and run on an IBM PC compatible microcomputer. Statistical analysis were performed using Statcalc (A. Lee, P. McInerney, and P. Mullins, Copyright © 1984) and an Apple IIe desk computer.

All kinetic parameter evaluations were done by systematically testing different model equations corresponding to either one or two Michaelian, saturating components working in the presence or absence of a nonspecific component. Explicit weighting of the data points was performed using the SD of regression when the initial rates were determined by linear-regression analysis or using the SE of the triplicate measurements when analyzing the one time point approach. In both cases, the robust weighting option of the "Enzfitter" program was used in order to minimize the contribution of possible outliers to the final fit. Both the goodness of fit of the model and the discrimination between possible alternative models was based on the criteria defined by Mannervik [28]: (*i*) convergence; (*ii*) parameter values; and (*iii*) residual plots. All residual plots shown in the figures are drawn relative to the substrate concentrations.

III. Results

A. Purity of Brush-Border Membrane Vesicles

The protein content as well as sucrase activity have been measured in both the mucosal homogenates and the vesicle fractions from two different individuals. As shown in Table 1, 0.9 to 1% of the total proteins were recovered in the vesicle fractions and sucrase activity was enriched by 9- to 12-fold in the two vesicle preparations. These results compare quite closely with previous determinations in adult human jejunum [23], but are about half of those of Harig et al. [13] in terms of sucrase activity. It should be noted, however, that our preparation appears free of contamination by functional basolateral membrane vesicles since glucose uptake was completely inhibited by phlorizin, a specific inhibitor of the brush-border, Na^+-dependent D-glucose transport activity (*see* Figs. 6 and 7).

B. Stability of Brush-Border Membrane Vesicles

Since uptake experiments usually last for a few hours, we have first verified the stability of the human brush-border membrane vesicles by measuring the initial rates of 50 μM D-glucose uptake every 2 hr over a 6-hr period. As shown in Table 2, the initial rates of D-glucose transport remained constant for at least 6 hr following their preparation. All of the experiments reported in the following studies have been performed within this time frame.

C. Linearity of D-Glucose Transport with Protein Concentrations

Brush-border membrane vesicles have been resuspended at different protein concentrations ranging from 2 to 21 mg per ml. As demonstrated in Fig. 1, the initial rates of transport determined at a 50-μM D-glucose concentration were linear over this range of protein concentrations. In the following, all experiments have been performed using concentrations of 9–11 mg of brush-border membrane proteins per ml of resuspension medium.

D. Time Courses of D-Glucose Uptake at Varying Unlabeled Substrate Concentrations Using the FSRFA

In the first series of experiments to be reported here, human brush-border membrane vesicles were injected into different incubation media containing a

Table 2. Stability of BBMVs from adult human jejunum

Time after BBMV preparation (hr)	Initial rate of D-glucose uptake (pmoles · sec^{-1} · mg $protein^{-1}$)
0	33.8 ± 1.0[a]
2	33.8 ± 2.5
4	35.2 ± 1.9
6	38.1 ± 1.6

[a] Value ± SD determined by linear-regression analysis performed on the linear portion of the uptake time curve (nine points over 4.5 sec). BBMVs were resuspended in 50 mM Tris-HEPES buffer (pH 7.5), 0.1 mM $MgSO_4$, 100 mM KCl, 200 mM choline chloride, 125 mM mannitol, and 5 μM valinomycin. Final concentrations in the incubation medium were: 50 mM Tris-HEPES buffer (pH 7.5), 0.1 mM $MgSO_4$, 100 mM KCl, 192 mM NaCl, 8 mM choline chloride, 125 mM mannitol, 0.5 mM amiloride, 50 μM D-glucose, and 4 μM D-(^{3}H)glucose.

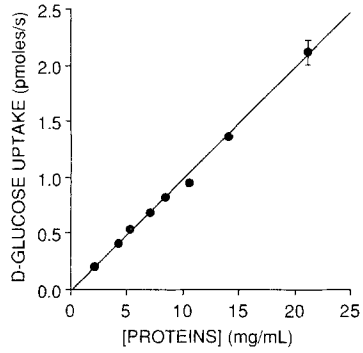

Fig. 1. Relationship between initial rates of D-glucose uptake and membrane protein concentrations. Brush-border membrane vesicles were resuspended in 50 mM Tris-HEPES buffer (pH 7.5), 0.1 mM $MgSO_4$, 100 mM KCl, 200 mM choline chloride, 10 mM mannitol, and 5 μM valinomycin. Final concentrations in the incubation media were: 50 mM Tris-HEPES buffer, 0.1 mM $MgSO_4$, 100 mM KCl, 192 mM NaCl, 8 mM choline chloride, 10 mM mannitol, 0.5 mM amiloride, and 50 μM D-(^{14}C)glucose. The initial rates of D-glucose uptake (±SD of regression) have been determined by linear-regression analysis over the linear portion of the uptake time curves generated with the FSRFA (nine time points equally spaced over 4.5 sec). When not shown, the error bars were smaller than the symbols used. Linear-regression analysis over the data points gave a *y* intercept, slope and correlation coefficient of −0.014 ± 0.020 pmol · sec^{-1}, 0.099 ± 0.002 pmol · sec^{-1} · mg $protein^{-1}$, and 0.998, respectively

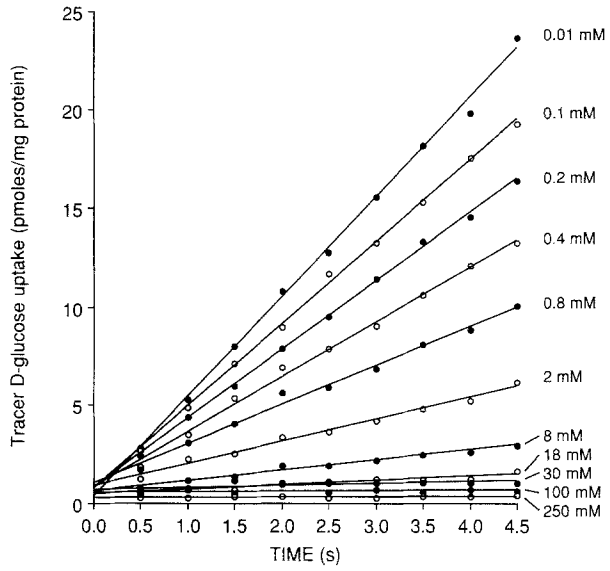

Fig. 2. Time courses of tracer D-glucose uptake at varying unlabeled substrate concentrations. Vesicles were resuspended in 50 mM Tris-HEPES buffer (pH 7.5), 0.1 mM $MgSO_4$, 100 mM KCl, 200 mM choline chloride, 300 mM mannitol, and 5 μM valinomycin. Final concentrations in the incubation media were: 50 mM Tris-HEPES buffer (pH 7.5), 0.1 mM $MgSO_4$, 100 mM KCl, 192 mM NaCl, 8 mM choline chloride, 0.5 mM amiloride, 4 μM D-(^{3}H)glucose, and increasing concentrations of unlabeled D-glucose (from 0 up to 250 mM) in the incubation medium. Osmolarity of the resuspension buffer was maintained with mannitol to keep a fixed total sugar concentration of 300 mM. Lines shown are the best fit lines at each substrate concentration as determined by linear regression over the nine time points

constant amount of radioactive substrate (0.4 μM ^{3}H-D-glucose) and 23 concentrations of unlabeled D-glucose ranging from 0 to 250 mM. The incubation media also contained a constant concentration of 0.4 mM ^{14}C-D-mannitol which serves as a space marker and could thus allow for water space (dead space) correction of the uptake data. Such a protocol was validated in the preceding paper [5] by showing: (*i*) that the water space determination in our system is independent of the use of either ^{3}H or ^{14}C isotopes and is equivalent when using either D-glucose or D-mannitol; and (*ii*) that D-mannitol uptake in human brush-border membrane vesicles is insignificant up to 10-min incubation with vesicles.

Figure 2 represents the uptake time courses of 4 μM tracer D-glucose at varying unlabeled substrate concentrations over a 4.5-sec incubation period with samplings from the same incubation medium at 0.5-sec time intervals. For clarity, only 11 concentra-

tions representative of the whole range are shown. In Fig. 2, the space marker correction was not performed although values in the ^3H channel were corrected for the ^{14}C crossing over. Figure 2 clearly shows that the uptake time courses are linear over the chosen time range for all substrate concentrations, thus extending our results of the preceding paper [5] to the whole range of substrate concentrations. Also, it is obvious from Fig. 2 that the initial rates of tracer D-glucose uptake decrease with increasing concentrations of unlabeled substrate. All the regression lines intercept the *y*-axis at a mean value ± SD of 0.61 ± 0.27 pmol · mg protein^{-1} (n = 23). Accordingly, this background value appears independent of the concentration of unlabeled substrate added to the incubation medium. From Fig. 2, it is finally important to note that the slopes of the regression lines were not significantly different from zero over 18-mM substrate concentration.

When the data of Fig. 2 were corrected for the mannitol space, a similar picture emerged, and for this reason, these results are not shown. The following remarks however apply: (*i*) identical slopes were obtained for each substrate concentration, thus showing that the corrected data is actually a family of lines parallel to those shown in Fig. 2; and (*ii*) that the mean intercept value ± SD was 0.13 ± 0.24 pmol · mg protein^{-1} (n = 23). Obviously, this value is not different from zero, thus showing that the mean positive intercept in Fig. 2 is fully accounted for by nonspecific, nonsaturable trapping of substrate at zero time in a dead space. For these reasons, ^{14}C-D-mannitol was not used anymore in any of the following experiments.

E. Kinetic Parameter Determination when Using the FSRFA

Obviously, one faces a major problem when trying to analyze the data of Fig. 2 by using the classical v *versus* S plot since zero initial rates are obtained at the high substrate concentrations. Accordingly, when trying to express the initial rates of transport relative to the total substrate concentrations by dividing each of the slope values from Fig. 2 by the corresponding specific activity of the tracer at each substrate concentration, zero values will also be obtained and the data cannot be evaluated over the whole range of substrate concentrations studied. This problem can, however, be easily solved without dropping any of the 23 concentrations of Fig. 2 by considering that the data, as presented, does correspond to a displacement of radioactive substrate from (a) specific site(s). A v_T (rate at tracer concentration) *versus* S (unlabeled substrate concentration) plot can thus be constructed as usually done in binding studies. The equations to be used for the analysis of such curves are easily derived by considering that the rates at pure tracer concentrations (T) would obey the Michaelis-Menten Eq. (1) where V_{max} and K_m have their usual meanings:

$$v_T = (V_{max} \cdot (T))/(K_m + (T)). \quad (1)$$

The cold substrate (S) will thus behave as a competitive inhibitor respective to the tracer flux and the Michaelis constant of Eq. (1) has to be replaced by K'_m as defined by Eq. (2)

$$K'_m = K_m(1 + (S)/K_m). \quad (2)$$

Equation (1) thus becomes

$$v_T = (V_{max} \cdot (T))/(K_m + (T) + (S)). \quad (3)$$

Equation (3) is perfectly suitable for nonlinear-regression analysis with (S) as the independent variable and with V_{max} and K_m as the dependent variables. The variable (T) is defined as a prompt constant and needs to be given to the computer before fitting Eq. (3) to the data points. The following remarks need to be considered: (*i*) Eq. (3) is actually equivalent to Eq. (1) in terms of the number of unknowns to be found by iteration; (*ii*) Eq. (3) explicitly implies a separation of the variables (S) and (T), or, stated otherwise, (S) should not be taken as the total substrate concentration; (*iii*) the analysis for more than one saturable component can be done by adding as many Eqs. (3) as needed in which different V_{max} and K_m terms can identify each saturable component; and (*iv*) nonspecific uptake components can also be easily introduced into Eq. (3) by adding a constant term since nonspecific components are by definition not displaced by cold substrate concentrations.

The initial rates of D-glucose uptake at different concentrations of unlabeled substrate have thus been analyzed by nonlinear regression using Eq. (3). As shown in Fig. 3*A*, a single system with a K_m of 0.59 ± 0.04 mM and a V_{max} of 718 ± 40 pmol · sec^{-1} · mg protein^{-1} seems to account for the data of Fig. 2, since the residual plot (inset of Fig. 3*A*) shows a random distribution of the data points around the zero mean. Models of either two sites or one site plus diffusion were rejected on the basis of either divergence or negative parameter values. As presented, however, it is clear that Fig. 3*A* does not allow for an easy visual appraisal of the goodness of fit. This can be largely improved by replotting the same data using a log scale in substrate concentrations as shown in Fig. 3*B*. Similar experiments have

Table 3. Kinetic parameters of Na^+-D-glucose cotransport system in adult human jejunum

Individual	20°C		35°C		
	K_m (mM)	V_{max} (pmol · sec^{-1} · mg $protein^{-1}$)	K_m (mM)	V_{max} (pmol · sec^{-1} · mg $protein^{-1}$)	K_d (pmol · mg $protein^{-1}$) at 4 μM tracer
#1	0.59 ± 0.04	718 ± 40	0.64 ± 0.09	2,074 ± 306	0.67 ± 0.29
#2	0.86 ± 0.07	729 ± 47	0.66 ± 0.06	1,833 ± 153	0.37 ± 0.10
#3	0.79 ± 0.11	968 ± 31	ND	ND	ND
Mean ± SD	0.75 ± 0.14	805 ± 141	0.65 ± 0.001	1,954 ± 170	0.52 ± 0.21

Experimental conditions were as described in the legend to Fig. 2.
ND = not determined.

been repeated on two other samples with essentially the same results, as reported in Table 3.

F. Comparison between the Dynamic and the One Time Point Approaches

Obviously, the results described above using the FSRFA are quite different from those usually obtained with the one time point approach, particularly as to the presence of a diffusive component in the uptake process. Such a one time point approach was thus simulated with the FSRFA as described in Materials and Methods and using brush-border membrane vesicles from individual #2 (Table 3). Also, in this set of experiments, D-glucose uptake has been evaluated over a smaller range of concentrations (18 concentrations from 4 μM to 50 mM) since saturation over this range is expected from Figs. 2 and 3.

As shown in Fig. 4, when the v over S plot was constructed from the 3-sec uptake values, the total uptake curve did not saturate over the range of concentrations studied. When analyzed by nonlinear regression, a single saturable system with a K_m of 0.81 ± 0.07 mM and a V_{max} of 642 ± 60 pmol · sec^{-1} · mg $protein^{-1}$ was obtained; a nonspecific component with a K_d of 1.30 ± 0.03 nmol · sec^{-1} mg $protein^{-1}$ · mM^{-1} was also determined (parameters ± SD regression). Attempts to fit either two sites or two sites plus diffusion equations to these data failed.

Applying Eq. (3) to these data, as shown in Fig. 5*A*, gave kinetic parameters similar to those determined above (*see* Table 4). A constant term of 5.2 ± 0.1 pmol · sec^{-1} · mg $protein^{-1}$ at 4 μM tracer concentration, which corresponds to 1.30 ± 0.03 nmol · sec^{-1} · mg $protein^{-1}$ · mM^{-1} was also obtained. In contrast, as shown in Fig. 5*B*, this nonspecific uptake component was no longer observed when the initial rates of D-glucose uptake were determined by linear regression over nine points and plotted according to the same Eq. (3), although kinetic parameter values were similar (Table 4).

As usually done using the one time point approach, the nonspecific uptake component was estimated by measuring D-glucose uptake in the presence of 1 mM phlorizin in the incubation medium. Under these conditions, the plot of D-glucose uptake as a function of increasing substrate concentrations gave a straight line with a slope of 1.3 nmol · sec^{-1} · mg $protein^{-1}$ · mM^{-1} (Fig. 6). However, as shown in Fig. 7, no correlation between tracer D-glucose uptake and time could be found over 4.5 sec in the presence of phlorizin and various concentrations of unlabeled substrate. The mean uptake value so determined was 4.7 ± 1.1 pmol · mg $protein^{-1}$ at 4 μM tracer concentration (1.2 ± 0.3 nmol · mg $protein^{-1}$ · mM^{-1}, n = 18). It is noteworthy that, in the absence of phlorizin, all uptake time curves intercepted to the same point on the y-axis (as shown in Fig. 2 for individual #1). The calculated mean value of this intercept actually represented 5.7 pmol · sec^{-1} · mg $protein^{-1}$ at 4 μM tracer (1.4 ± 0.04 nmol · mg $protein^{-1}$ · mM^{-1}, n = 18, *data not shown*).

In order to isolate the saturable component(s) of D-glucose uptake, the total uptake curve obtained at 3 sec (Fig. 4) has been corrected for the nonspecific uptake component determined in the presence of phlorizin (Fig. 6). As can be seen in Fig. 8*A*, very high dispersion in the data points is obtained for the highest concentrations of substrate and negative values are also generated. Plotting these data according to Eadie-Hofstee generates Fig. 8*B*. It should be noted that negative uptake values, as nonsensical as they might seem, have not been removed from the data of Fig. 8*A* and *B* since generated from points that do not need to be removed when the analysis is conducted according to Eq. (3) (*see* Fig. 5*A*). Using the kinetic parameters determined from the displacement curve of the same data (Fig. 5*A*), one can appreciate that these values adequately describe the v over S plot in the lower range of D-

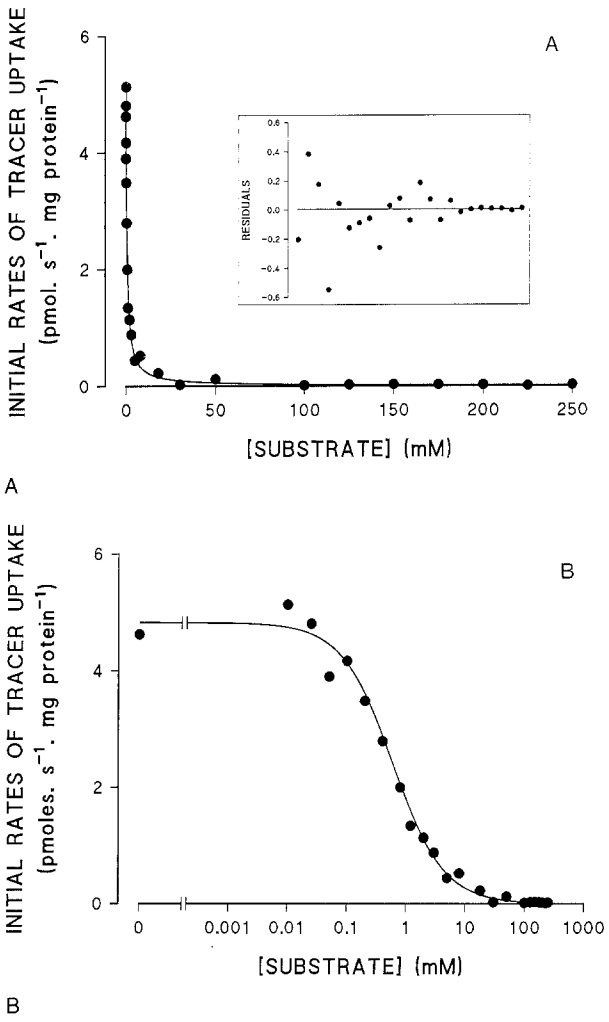

Fig. 3. Kinetic parameter determination of D-glucose transport using the FSRFA. Experimental conditions were as described in the legend to Fig. 2. The slopes of the regression lines, representing the true initial rates of transport, were plotted against the unlabeled substrate concentrations, and the data were analyzed according to Eq. (3) as described in the text. (*A*) Direct plot. (*B*) Log (*S*) plot. Nonlinear-regression analysis over the data points gave K_m and V_{max} values of 0.59 ± 0.04 mM and 718 ± 40 pmol · sec^{-1} · mg $protein^{-1}$, respectively. The residual plot of the fitted data is illustrated in the inset of *A* and shows random distribution around a zero mean. It should be noted that the SD of regression on the initial rate measurements were smaller than the symbols used

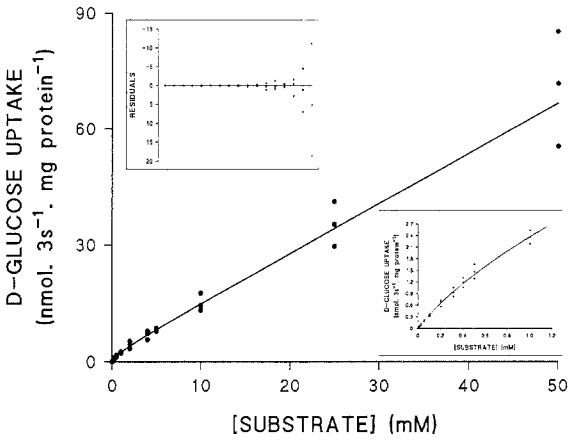

Fig. 4. Kinetic parameter determination of D-glucose transport from simulation of the one time point approach performed as described in Materials and Methods. Vesicles were resuspended in 50 mM Tris-HEPES buffer (pH 7.5), 0.1 mM $MgSO_4$, 100 mM KCl, 200 mM choline chloride, 125 mM mannitol, and 5 μM valinomycin. Final concentrations in the incubation media were: 50 mM Tris-HEPES buffer (pH 7.5), 0.1 mM $MgSO_4$, 100 mM KCl, 192 mM NaCl, 8 mM choline chloride, 0.5 mM amiloride, 4 μM D-(^{3}H)glucose, and variable concentrations of unlabeled D-glucose (from 0 up to 50 mM). Osmolarity was kept constant by adjusting the total sugar concentration with D-mannitol. Nonlinear-regression analysis over the data points gave the following kinetic parameters: K_m = 0.81 ± 0.07 mM, V_{max} = 642 ± 60 pmol · mg $protein^{-1}$, and K_d = 1.30 ± 0.03 nmol · sec^{-1} · mg $protein^{-1}$ · mM^{-1}, respectively. Points are mean ± SD from triplicates obtained at 3 ± 0.25 sec. Inset at top left shows the residual plot of the fitted data points while the inset at bottom right is an enlarged view of the *v versus S* plot which covers the lower range of substrate concentrations

glucose concentration (Fig. 8*A*, *inset*), and that, should the most deviant points be removed, the line drawn over the Eadie-Hofstee plot (Fig. 8*B*) could adequately describe the data. However, when analyzing the Eadie-Hofstee plot by linear regression, the kinetic parameters obtained are quite different (K_m: 0.61 ± 0.16 mM; V_{max}: 550 ± 93 pmol · sec^{-1} · mg $protein^{-1}$). Furthermore, removing the two negative values, one can generate an other set of parameters (K_m: 1.58 ± 0.48 mM; V_{max}: 1237 ± 271 pmol · sec^{-1} · mg $protein^{-1}$). It should also be pointed out that this plot could suggest the presence of more than one saturable component since the large dispersion of data points in the high substrate concentration range contribute to an overall impression of nonlinearity.

G. Kinetic Parameters of D-Glucose Transport at 35°C

All experiments reported so far, which demonstrate the presence of a unique transporter in adult human brush-border membrane vesicles, have been performed at 20°C. Since recent data in the guinea pig suggest a temperature activation effect on the visibility of a second site or carrier [7], we have tested this possibility in human membranes. Fifty μM D-glucose uptake has been first evaluated as a function of time at both 20 and 35°C. As shown in Fig. 9, the higher temperature produced the following changes: (*i*) the

Table 4. Summary of kinetic parameters estimated by nonlinear-regression analysis

Approach	K_m (mM)	V_{max} (pmol · s^{-1} · mg $protein^{-1}$)	K_d (nmol · mg $protein^{-1}$ · mM^{-1})
v over S plot at 3 sec	0.81 ± 0.07[a]	642 ± 60	1.30 ± 0.03
Displacement curve at 3s	0.82 ± 0.08	653 ± 61	1.30 ± 0.03
Displacement curve using the dynamic approach	0.86 ± 0.07	729 ± 47	—

[a] Brush-border membrane vesicles were prepared from individual #2 (Table 3). See Results, section F for more details.
[a] Value ± SD of regression.

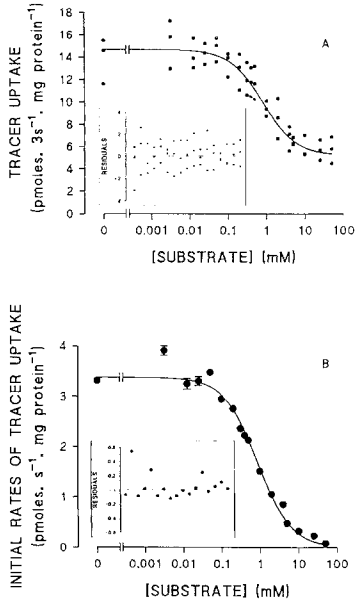

Fig. 5. Comparison of the displacement curves for D-glucose transport corresponding to the simulation of the one time point approach (*A*) or to the true initial rate determinations with the FSRFA (*B*). Experimental conditions were as described in the legend to Fig. 4. Data were analyzed by nonlinear-regression according to Eq. (3) with (*A*) or without (*B*) a constant term and plotted against log (*S*) as described in the text. When not shown, the SD were smaller than the symbols used. The kinetic parameters are listed in Table 4. Residual plots of the fitted data are shown in the insets

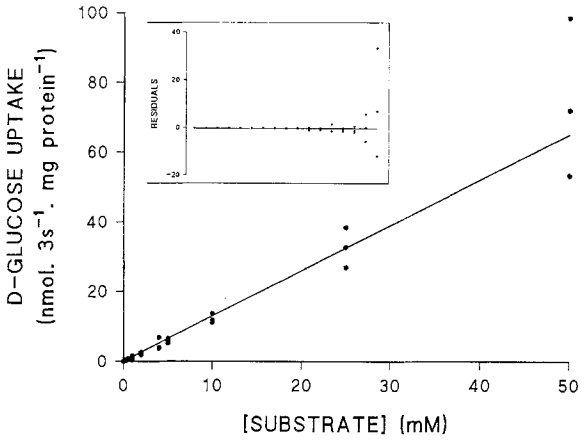

Fig. 6. Nonspecific uptake component as estimated from the simulation of the one time point approach. Experimental conditions were as described in the legend to Fig. 4 except for the presence of 1 mM phlorizin in the incubation medium. D-glucose uptake was plotted as a function of increasing substrate concentrations in the incubation media. Linear-regression analysis over the data points gave slope and intercept values of 1.3 ± 6.10^{-4} nmol · sec^{-1} · mg $protein^{-1}$ · mM^{-1} and 0.23 ± 6.10^{-3} nmol · 3 sec^{-1} · mg $protein^{-1}$, respectively. The inset shows the residual plot of the fitted data

peak of the overshoot is shifted to the left on the time axis, so the maximum overshoot value is recorded around 20 sec at 35°C instead of 60 sec at 20°C (*see also* ref. [5] for a more complete time course at 20°C); (*ii*) the height of the overshoot is decreased at 35°C as compared to 20°C, an effect which seems related to a faster efflux rate at the higher temperature, but which could also involve *trans* Na^+ inhibition [8, 33] since higher rates of Na^+ entry are expected at higher temperatures; and (*iii*) the initial rate of D-glucose uptake is increased at 35°C, but the period of initial linearity is greatly reduced. Thus, in the following, the initial rates of D-glucose uptake have been determined by using nine time points equally spaced over 2.7 sec. Brush-border membrane vesicles obtained from individual #2 (*see* Table 3) have been used for kinetic parameter determination. Uptake time courses similar to Fig. 2 were generated (*not shown*) and linearity over the selected time range was assessed. All regression lines also intercepted to the same point on the y-axis with mean value of 0.85 ± 0.37 pmol · mg $protein^{-1}$ at 4 μM tracer (n = 18). Also, in contrast to the situation at 20°C (*see* Fig. 2), finite slope values were obtained for the highest substrate concentrations tested. The initial rate data obtained has been analyzed by nonlinear regression using Eq. (3) to which a constant term was added and the results are shown in Fig. 10. As shown from the residual plot (inset of Fig. 10),

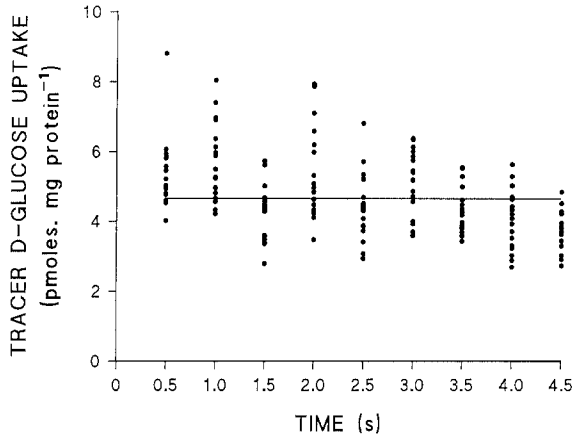

Fig. 7. Uptake time course of D-glucose in the presence of 1 mM phlorizin in the incubation medium as determined using the FSRFA. Experimental conditions were as described in the legend to Fig. 4. D-glucose uptake is expressed relative to the tracer concentration of substrate (4 μM) and was measured at 18 different concentrations of unlabeled substrate (from 0 to 50 mM). The mean uptake value was 4.7 ± 1.1 pmol · mg protein^{-1}

these data are perfectly compatible with the presence of a single saturable component with Michaelis constant similar to the one found previously for the same individual at 20°C (*see* Table 3). However, the V_{max} estimated at 35°C was 2.5-fold higher. Furthermore, a small nonspecific component, which in this case should represent passive diffusion of substrate into the vesicles, demonstrated a significant contribution to the total uptake data with a value ± SD of regression of 0.37 ± 0.10 pmol · sec^{-1} · mg protein^{-1} at a 4 μM tracer concentration. It should be noted that more complex model equations including two saturable components with or without a nonspecific pathway could not be fitted to these data. Similar results were also obtained with individual #1 (*see* Table 3).

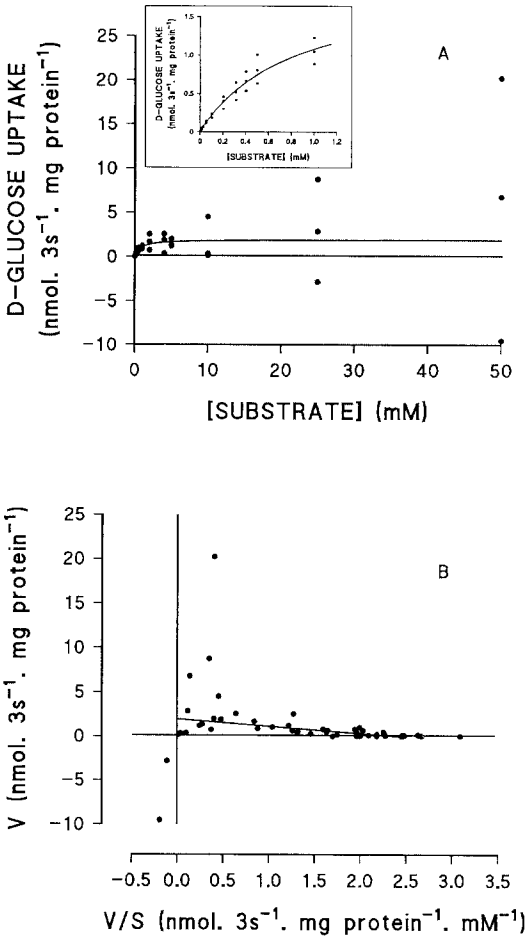

Fig. 8. Linearization of the specific transport component from the D-glucose uptake data corresponding to the one time point approach. (*A*) *v versus S* plot generated after subtraction of the nonspecific component estimated in Fig. 6 from the total uptake data of Fig. 4. An enlarged view of D-glucose uptake in the low range of substrate concentrations is shown in the inset. (*B*) Eadie-Hofstee plot of the same data. In both cases, the drawn line corresponds to the kinetic parameters determined from the analysis of the displacement curve from the same data as reported in Fig. 5*A*

IV. Discussion

In these studies, we have used a recently developed fast sampling, rapid filtration apparatus (FSRFA) [5] to study the kinetic characteristics of Na^+-coupled D-glucose transport in adult human jejunal brush-border membrane vesicles. These membrane preparations have been controlled in terms of purity, stability with time, and linearity of uptake as a function of protein concentrations. An adequate interpretation of the kinetic data does rely on the validity of these basic conditions, which were demonstrated to be fulfilled in these preparations.

As reported in the preceding paper [5], the FSRFA allows for the estimation of the initial transport rates by linear-regression analysis over multiple time points obtained within the first few seconds of incubation. As illustrated in Fig. 2, D-glucose uptake at 20°C is linear during 4.5 sec over the whole range of substrate concentrations tested. This initial linearity period is however decreased to 2.7 sec when increasing the temperature to 35°C. It has been argued by Hopfer [14–16] and more recently by Kimmich [20] that zero-*trans* gradient conditions, because of their transient nature, could lead to variable conditions when changing the concentrations of either substrate or Na^+ within an experiment. However, it has also been concluded that the steady-state

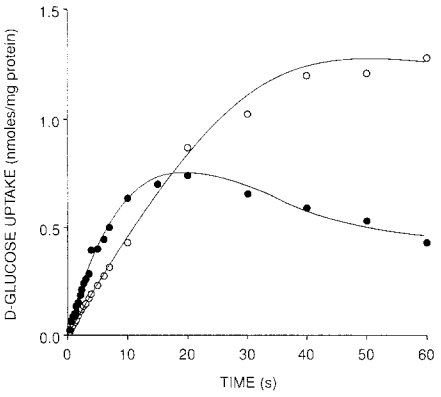

Fig. 9. Comparison of the uptake time courses of D-glucose at 20°C (○) and 35°C (●) in human jejunal brush-border membrane vesicles. Vesicles were resuspended in 50 mM Tris-HEPES buffer (pH 7.5), 0.1 mM $MgSO_4$, 100 mM KCl, 200 mM choline chloride, and 5 μM valinomycin. Final concentrations in the incubation media were: 50 mM Tris-HEPES buffer (pH 7.5), 0.1 mM $MgSO_4$, 100 mM KCl, 192 mM NaCl, 8 mM choline chloride, 0.5 mM amiloride, and 50 μM ^{14}C-D-glucose. The data points were generated using the FSRFA

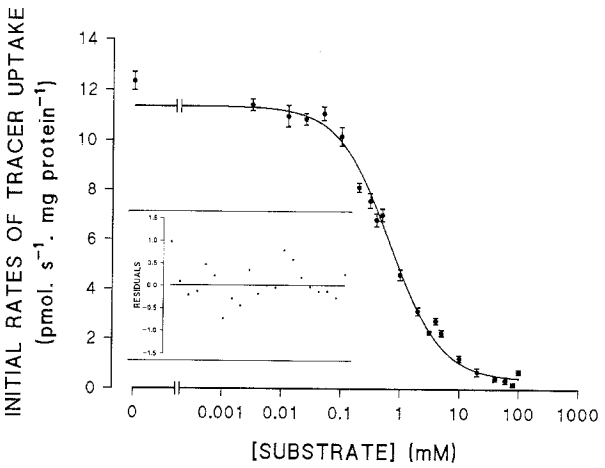

Fig. 10. Displacement curve for the D-glucose transport data at 35°C. The compositions of the resuspension and incubation media were as described in the legend to Fig. 2. Concentrations of unlabeled substrate were varied from 0 to 100 mM. Initial rates of transport (±SD of regression) were estimated by linear-regression analysis using nine time points equally spaced over 2.7 sec. Nonlinear-regression analysis according to Eq. (3) in the text with addition of a constant term gave the following kinetic parameters: K_m = 0.66 ± 0.06 mM, V_{max} = 1833 ± 153 pmol · sec^{-1} · mg protein^{-1}, and constant = 0.37 ± 0.10 pmol · mg protein^{-1}. The residual plot of the fitted data is shown in the inset

assumption should apply under gradient conditions whether or not the membrane potential and/or the ion and substrate gradients collapse with time, provided that initial linearity, which represents in itself a sufficient warranty as to the existence of a prevailing steady state, is demonstrated [6]. This statement is particularly relevant in the context of the present paper since the behavior predicted by Hopfer [14–16] and Kimmich [20] from these variable conditions would be the generation of an artefactual heterogeneity in transport sites. It is clear from our studies, however, that the data at both 20 and 35°C are compatible with a single Michaelian behavior (*see* Figs. 3 and 10). From these results and from the demonstration that Na^+-dependent D-glucose transport is completely inhibited by 1 mM phlorizin (*see* Fig. 7), it can thus be concluded that a unique and fully Na^+-dependent D-glucose carrier is present in the adult human brush-border membrane vesicles, at least when estimated under 0-mV voltage-clamped conditions and at 200 mM outside Na^+. However, since the situation in the adult tissue appears quite different from that reported previously for the human fetal jejunum [25, 26], a complete characterization in terms of substrate specificity and/or inhibitor sensitivity should be done before a definitive conclusion can be drawn concerning the existence of one or multiple Na^+-D-glucose cotransport system(s) (or site(s)) in the adult human jejunum. More studies would thus be needed to fully characterize Na^+-D-glucose cotransport in these membranes.

Our conclusions thus conflict with those recently reported by Harig et al. [13]. Since their studies have also been performed with jejunal brush-border membranes vesicles isolated from healthy organ donors and since their kinetic measurements have been done under 0-mV voltage-clamped conditions, it would appear that an explanation for these discrepancies should first be searched among other experimental conditions. That the lower outside Na^+ concentration of 100 mM used by these authors could account for the discrepancy seems unlikely since the glucose transporter is far from being saturated at these two concentrations (C. Malo, *unpublished*). Also, the studies of Harig et al. [13] have been performed at 25°C, whereas our first measurements have been done at 20°C (*see* Fig. 2). Might this difference in temperature have been responsible for the different results obtained? This question is well grounded when considering the work of Brot-Laroche et al. [7] who have reported the presence of two saturable components for D-glucose uptake in the guinea-pig small intestine at 35°C. The low-affinity *S*-2 system was found to be active between 25 and 35°C, while it could not be distinguished from diffusion at lower temperature [1]. Therefore, our kinetic studies were repeated at 35°C, taking advantage of the thermoregulated incubation chamber of the FSRFA [5]. The results obtained with two different individuals (*see* Fig. 10 and Table 3) were still

compatible with a single system with K_m values similar to the ones obtained at 20°C (Table 3). However, the V_{max} values were 2.5–2.9 times higher, thus suggesting a faster turnover rate of the carrier at this temperature. Obviously, species differences could probably account for the discrepancies between our results and those of Brot-Laroche et al. [7], but this explanation does not hold when comparing with the results of Harig et al. [13].

It would thus appear that the main difference between our conclusions and those of Harig et al. [13] resides in the analytical approaches which were used to separate the different uptake components and to determine their kinetic parameters. In our studies, we have combined a dynamic approach and a nonlinear-regression analysis to attain these goals, whereas a one time point approach coupled to linear transformation of the corrected data has been applied by Harig et al. [13]. We thus compared these two approaches in order to evaluate the potential problems which could influence the interpretation of kinetic data. It should be stressed that the approach chosen by Harig et al. [13] is actually the most current one in the field of transport, and thus, that the discussion which follows should apply to other studies as well.

As recently reviewed [6] and discussed [5], substrate uptake into brush-border membrane vesicles is a composite of different components, both specific and nonspecific, that occur simultaneously during the incubation process. Obviously, their respective contributions to the overall uptake might need to be evaluated and separated for meaningful transport kinetic analysis and data interpretation. However, when one is interested in carrier-mediated process(es) only, such detailed studies might not be necessary, and it is the main thrust of the following discussion to demonstrate that the most reliable kinetic analyses are to be expected when avoiding too many data corrections.

As shown in Fig. 2, when the uptake data are performed at constant tracer concentration as is often the case in transport experiments, the slopes of the uptake time courses decrease with increasing concentrations of unlabeled substrate. The meaning of this experiment is quite clear, since it demonstrates that low cpm values are measured at high substrate concentrations. Accordingly, this sets up the limit of the radiotracer approach, since finite values of fluxes will become more and more difficult to dissociate from the background. This is particularly well exemplified by the data of Fig. 2 where, at high D-glucose concentrations, there is no uptake over the background value and the slopes become equal to zero. It could be argued that the data of Fig. 2 constitute quite a particular case since simple diffusion of glucose cannot be measured at 20°C in human brush-border membrane vesicles [5] (*see also* Fig. 7). While true, it should nonetheless be noted that the general trend in cpm measurements is not particular to the human vesicles and that the same behavior has been observed at 35°C where passive diffusion of D-glucose was measured. However, using the one time point approach, a *v versus S* plots can always be constructed (*see* Fig. 4). In this case, the low cpm values obtained at high concentrations of substrate are multiplied by a large dilution factor (up to 12,500 at 50 mM) which takes into account the specific activity of the tracer at each substrate concentration; this procedure generates an alteration in the error structure of the data, as can easily be verified by comparing Figs. 4 and 5*A*. Also, it is quite obvious from the comparison of these two figures that the graphical representation of Fig. 5*A* gives some information which cannot be visually assessed from Fig. 4: (*i*) it clearly shows that a plateau was obtained at both ends of the substrate concentration range, and therefore, the whole range of significant concentrations was actually covered; (*ii*) it illustrates the sensitivity of the assay and gives a direct appraisal of the signal over noise ratio, whereas the *v versus S* plot of Fig. 4 gives the false impression that the nonspecific component was predominant over the saturable one; and (*iii*) it does not bring forth the nature of the nonspecific component which is generally assumed to be diffusion in the *v versus S* plot since showing linearity in substrate concentrations. It should also be pointed out that the analysis according to the displacement curve allows for an appreciable time saving. Actually, it is possible with this type of analysis to directly use the raw cpm data, the only calculation needed being the transformation of V_{max} in conventional units. It can thus be concluded that the displacement curve could advantageously replace the *v versus S* plot without any loss in terms of kinetic parameter estimation while saving a lot of time in the processing of the raw data for computational purpose and allowing a better appreciation of the real data. However, it should be pointed out that the kinetic parameters determined from either the *v versus S* plot or the displacement curve are actually identical when nonlinear-regression analysis and equivalent weighting routines are applied (Table 4).

From the above discussion, it would appear that the nonspecific components, whether representing true passive diffusion, nonspecific binding, uncorrected water space, or a mix of any of these, can be estimated at once when using a nonlinear-regression analysis without requiring for any data correction. The situation is however quite different if linear transformations of (a) Michaelis-Menten process(es)

are to be used. In fact, assuming that linearity or nonlinearity of these plots should serve as a diagnostic test for the absence or presence of more than one transport site or protein, the data need to be corrected for all nonspecific components which, otherwise, would interfere with the linearization process itself. Different approaches have been proposed and used to correct for these nonspecific components.

(*i*) Correction for the dead space can be easily made by running a zero time point in the presence of vesicles where radioactive substrate and stop solution are added simultaneously. Obviously, such a practice implicitly assumes exact reproducibility from sample to sample. Such a hypothesis is actually not verified, considering D-glucose uptake measurements in the presence of phlorizin. In fact, the data from Fig. 7 does represent the output to be expected from the background correction suggested above since there is no measurable passive diffusion at 20°C in these brush-border membrane vesicles. What is actually obtained is a mean value ± SD which clearly shows that this value is not unique and error free. Furthermore, if the concentration of radiolabeled substrate is increased as a function of increasing concentration of unlabeled substrate in order to avoid too high isotopic dilution, an adequate correction for a nonspecific binding component can be more difficult to achieve. It should also be noticed that this background value may vary from one experiment to the other, as recorded in our own experiments (*see* Figs. 2 and 7). This difference can be attributed mainly to the filters, since we have noted that different batches of filters, even from the same source, give different background values. In the course of a single experiment, the same batch of filters has been consistently used.

(*ii*) A better way to correct for the dead space may be to use a "quenched" stop solution which contains a differentially labeled space marker or substrate analogue (usually an inactive stereoisomer), since such a correction records the situation on each filter individually. Again, in the absence of simple diffusion in human brush-border membrane vesicles at 20°C, the mannitol-corrected data of Fig. 2 (described in Results, section D) simulates such a situation. While the mean y intercept was now not significantly different from zero, it was nevertheless associated with a SD term which states quite clearly that such correction is not error free either. The problem could easily be solved when using the dynamic approach, since the regression lines could be forced through a zero intercept. However, it cannot be handled properly when using the one time point approach.

(*iii*) The other nonspecific component which needs to be corrected for when using linear transformations of (a) Michaelian process(es) is the simple, passive diffusion of substrate. Two properties of this uptake component, namely its linearity with increasing substrate concentrations and its insensitivity to inhibition by known transport inhibitors or substrate analogues have set up the strategies commonly used for its evaluation. The straight line usually obtained by constructing the corresponding *v versus S* plot is usually taken as evidence for the contribution of a diffusive component to total transport and its slope assimilated to the diffusion rate constant K_d. As straightforward as it might seem, our results clearly show that such a simple analysis can easily lead to erroneous conclusions, since simple diffusion cannot be dissociated from the other nonspecific components based on these criteria alone. This conclusion is best exemplified by considering the data of Figs. 4 and 6 which could argue for the presence of a quite large diffusional component in total transport when actually such a component could not be measured directly (*see* Fig. 7). So, what does represent the linear uptake component measured at 3 sec in the absence (Fig. 4) or in the presence of phlorizin (Fig. 6) in the incubation medium? Actually, the slope of this straight line has a value which is very close to the mean background value as estimated from Fig. 7 and also similar to the mean value of the common y intercepts as determined from the uptake time curves (1.4 ± 0.04 nmol · mg protein^{-1} · mM^{-1}, *data not shown,* but similar in form to Fig. 2). Obviously, when using the one time point approach, both the intercept (binding) and the slope (initial rate) components of the uptake time courses are included in the measured values, thus giving the general profile of the *v versus S* plot (Fig. 4). Indeed, the linear relationship observed in Fig. 6 is a strong evidence for the presence of a fast, nonspecific binding component. It can thus be concluded that the so-called diffusional component should be considered as an operational parameter only, since it may fail to give any meaningful information as to the real passive permeability of the membrane.

When both the background and the "diffusional" components have been estimated, one has to extract their contributions from the total uptake data in order to analyze the saturable component(s). Since both corrections are not error free, subtraction procedures which actually ignore the statistical figures do add noise to the experimental data points. While such a situation could be quite acceptable in the lower range of substrate concentrations where low nonspecific uptake values are subtracted from high total uptake ones, it should be stressed that it may not be adequate anymore for the higher range of substrate concentrations where specific and non-

specific uptake values tend to be of the same order of magnitude. This phenomenon is clearly illustrated in Fig. 8*A* where data are largely dispersed at high substrate concentrations, whereas the fit in the lower range of substrate concentrations is quite acceptable (*see* inset of Fig. 8*A*). The determination of kinetic parameters from such data is obviously less accurate. Furthermore, should all of the disturbing points be removed on the rationale (and quite valid in fact) that negative and/or zero values have no physiological meaning in the context of such experiments, it might become possible to artefactually create deformations in the Eadie-Hofstee plot (Fig. 8*B*) that could account for an extra linear component and/or a second site in some situations. It should also be pointed out that increasing the number of data points in the high *S* region and using unweighted-regression analysis (even nonlinear) could actually lead to resolution of such pseudo-uptake components. In this context and considering the fact that no errors are associated with the Eadie-Hofstee plot reported in Fig. 3 of the paper by Harig et al. [13], one may assume that unweighted regression was used for its analysis. That their second site might be due to an underestimation of the nonspecific uptake component(s) is a logical proposal that stems from our studies; this possibility could be easily tested by reanalyzing these data according to a nonlinear-regression procedure which, in all cases (*see* Figs. 4 and 5), bypasses the difficulty inherent to the correct estimation of the nonspecific uptake component.

In conclusion, our studies clearly demonstrate that great care should be taken when trying to analyze transport kinetic data using the rapid filtration technique. While it does not matter as to whether a dynamic or a one time point approach is chosen to generate the uptake data provided that a nonlinear-regression analysis with proper weighting of the data points is performed, it clearly appears that analytical procedures which rely on a linearization of Michaelian processes are endowed with a number of difficulties which cannot be easily evaluated (particularly when using the one time point approach) and could lead to erroneous interpretations of the data. More specifically, this approach should be avoided when trying to resolve multicomponent systems, since we have demonstrated that this approach could actually generate artefactual components due to the subtraction step which is inherent to this type of analysis. In this regard, the FSRFA proves to be very useful for the identification of the nonspecific uptake pathways and for the evaluation of their respective contribution to total uptake in a given system. As previously stated in biochemical and pharmacological studies [24], it is also the point of view of the authors that linear-regression analysis should not be applied to linearizing plots in transport studies as well. Finally, we would like to suggest a more general approach for the analysis of uncorrected transport data which makes use of a displacement curve instead of the usual *v versus S* plot, since we demonstrate that it is far superior in terms of rapidity and visual informations. Should these remarks be interpreted as meaning that there are no such things like second sites or different carrier proteins involved in the transport of different solutes? Obviously not, but they do point out weaknesses in current methods of data analysis which can be easily resolved and turned into a more powerful approach in trying to resolve such problems.

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