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A SIMPLE APPARATUS FOR PERFORMING SHORT-TIME (1—2 SECONDS) UPTAKE MEASUREMENTS IN SMALL VOLUMES; ITS APPLICATION TO D-GLUCOSE TRANSPORT STUDIES IN BRUSH BORDER VESICLES FROM RABBIT JEJUNUM AND ILEUM

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Summary

An automated procedure allows uptake measurements with incubation times as short as 0.5 s and with volumes of 10—20 μl. Using this technique the kinetic parameters K_m and V of D-glucose transport in brush border vesicles from rabbit small intestine could be determined from unidirectional fluxes. A comparison of the data obtained from jejunum and from ileum shows that the K_m for D-glucose is the same in both parts of the intestine, whereas the maximum flux is significantly larger in the jejunum.

Introduction

Fast transport processes in subcellular organelles often maintain their initial rates for only a few seconds or less. Due to an unfavourable surface/volume ratio, efflux of substrate from the vesicles and/or dissipation of a driving force and/or *trans*-effects quickly become important factors in the decreasing rate of net uptake. The sodium-dependent uptake of D-glucose and L-amino acids by brush border vesicles derived from intestine and kidney are fast processes subject to these limitations [1—8]: (a) D-Glucose equilibrates across the membrane with a half-filling time $t_{1/2}$ of 10—30 s when sodium is present on both sides of the membrane. (b) When a sodium gradient is applied to the brush border vesicles, the non-electrolyte can be accumulated inside the vesicles at a level several times that of its external concentration [2,4,6]. D-Glucose reaches the maximum concentration after about 45 s; however, deviation from linearity occurs as early as 3—5 s.

Abbreviation: HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid.

To date, the numerous studies of transport in brush border vesicles have been performed with incubation times of 10 s and longer [1—8] and, therefore, the measured transport rates may substantially differ from initial uptake rates. We were interested in studying true initial rates of transport and in determining more reliable figures for the macroscopic kinetic parameters of unidirectional flux such as K_m and V. Our initial 'short-time' incubation studies were started and stopped manually. The shortest time that gave reasonably reliable results was approx. 5 s. We considered using a stop-flow apparatus to automate the procedure. However, a stop-flow apparatus would require a large volume of vesicles, i.e., 100 μl—1 ml for each incubation, whereas the total yield of a single brush border preparation cannot usefully be suspended in more than 2 ml. Therefore, in order to perform incubations in the range of 0.5—5 s, using volumes of vesicle suspensions as small as 10 μl, we designed a simple apparatus which automatically controls the mixing of vesicles and substrate and the termination of the incubation. We report here the operation and the properties of the apparatus and present typical data that can be obtained with it.

Methods and Materials

Brush border vesicles were prepared from frozen rabbit intestine by the calcium method, as first described by Schmitz et al. [9] and modified by Kessler et al. [6]. The yield of one preparation, 20—30 mg of membrane protein, was suspended in about 1—2 ml of a solution containing 100 mM mannitol and 10 mM HEPES/Tris, pH 7.5. To initiate transport, 10 μl of vesicle suspension were rapidly mixed with 10 μl of a solution containing, in addition to 100 mM mannitol and 10 mM HEPES/Tris, pH 7.5, the labelled substrate (0.2—1 μCi) and the additions as described in the figure legends. All transport studies were performed at room temperature. The reaction was stopped by adding 2 ml of ice-cold 0.9% saline solution buffered with 1 mM Tris · HCl, pH 7.5. The solution was immediately filtered on a pre-wetted Sartorius filter (0.6 μm pore size). The filter was washed with 6 ml of the same solution. Blanks, accounting for the label trapped on the filter, were determined by adding the vesicle suspension and the solution containing the substrate separately to the stop solution.

Yeast β-fructosidase was purchased from Boehringer GmbH, Mannheim (EC 3.2.1.26). The enzyme was dissolved in a vesicle suspension, giving a final concentration of 10 mg membrane protein and 5 mg fructosidase per ml. 10 μl of this suspension were mixed with 10 μl of a 100 mM sucrose solution. Hydrolysis of sucrose was terminated by adding 2 ml of 200 mM Na_2HPO_4 solution. The solution was then heated at 100°C for 5 min. The liberated glucose was measured by the Tris-glucose oxidase method [10].

Sucrase was determined according to the method of Dahlqvist, using the Tris/glucose oxidase/peroxidase reagent [10].

All reagents were of highest purity available. D-[1-^{3}H]Glucose was purchased from Amersham Radiochemical Centre, Ltd., Bucks.; L-[2-(n)-^{3}H]methionine was purchased from New England Nuclear, Boston, Mass.

Results and Discussion

The apparatus we use consists of a timer, a shaker and a stop solution injector.

Shaker. The incubations are performed at room temperature (20 ± 2°C) in 5-ml clear polystyrene test tubes (diameter 13 mm, length 70 mm). The two drops of 10 μl each (vesicle- and substrate-containing medium) are placed close to each other at the bottom of the test tube, in line with the direction of shaking. The test tube is fitted in a sled connected to a solenoid-vibrator (frequency about 60 Hz). Mixing of the two drops is obtained by switching on the vibrator. The frequency and the amplitude of the shaking can be regulated and have to be properly adjusted in order to get rapid mixing without dispersion of the drops.

Stop solution injector (see Fig. 1). Compressed air, controlled by an electromagnetic valve (C) is used to eject 2 ml of stop solution from the siphon (A). For refilling the siphon, a reservoir of the stop solution (E) is placed above the siphon and connected to the latter by a silicon tubing, which can be compressed and opened by a solenoid (F). Both the siphon and the reservoir are surrounded by a mantle percolated with ice-cold water (B, D).

Filtration. After the injection of the stop solution, the contents of the test tube were immediately poured on the filter and washed as described under Methods and Materials. The time elapsed between stopping and the end of the washing was 10—15 s. In some experiments, this time was arbitrarily prolonged by 30 or 60 s in order to see how much substrate the vesicles lose after being diluted in the ice-cold stop solution. Plotting these values semilogarithmically and extrapolating them back to the actual time of stopping indicated that up to 20% was lost during the stop and wash procedure, when sodium was present on both sides of the membrane. However, if the internal sodium concentration was low, the loss was only about 5%. This latter condition obtains during the initial phase of all the incubations performed under an initial sodium gradient.

Time accuracy. The pilot instrument can only give an accurate time for the start and the stop pulse. The actual time of the incubation is certainly different

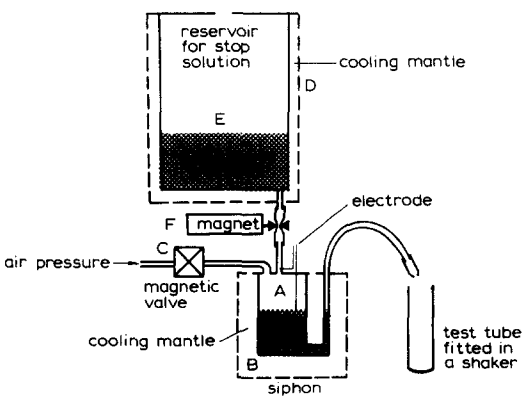

Fig. 1. Schematic diagram of the stop solution injector. For explanations see text.

from the chosen time for two reasons: first, time is required for complete mixing and second, time elapses between the opening of the siphon valve (C) and the arrival of the stop solution at the bottom of the test tube.

The time delay due to the addition of the stop solution was directly measured using electrodes placed in the bottom of the test tube. The times measured varied between 40 and 60 ms. This number depends on the air pressure and the geometry of the injector.

The time required for complete mixing cannot be measured electronically. If the drops are placed close enough together at the bottom of the tube, the drops probably fuse at the first stroke of the shaker (approx. 15 ms). The time for complete mixing is longer. In order to determine mixing time, we measured the onset of an enzymic reaction. Since the specific activities of the membrane-bound enzymes were too low, yeast β-fructosidase was added to the membrane preparation. (Fructosidase and membranes were incubated together in order to have comparable surface tensions of the drops as in transport measurements.) Hydrolysis of sucrose was determined at incubation times between 0.1 and 0.5 s and extrapolated back to the actual time of zero hydrolysis.Therefrom a mixing time of 70 ± 10 ms was determined.

D-Glucose transport in brush border membrane vesicles

In the presence of an initial sodium gradient, D-glucose is transiently accumulated by brush border vesicles. The underlying process, the coupled transport of Na^+ and D-glucose, has been found to be rheogenic [2,5]. Therefore, the uptake rate and the extent of accumulation depend on the size of the transmembrane potential, which in turn is partly determined by the membrane

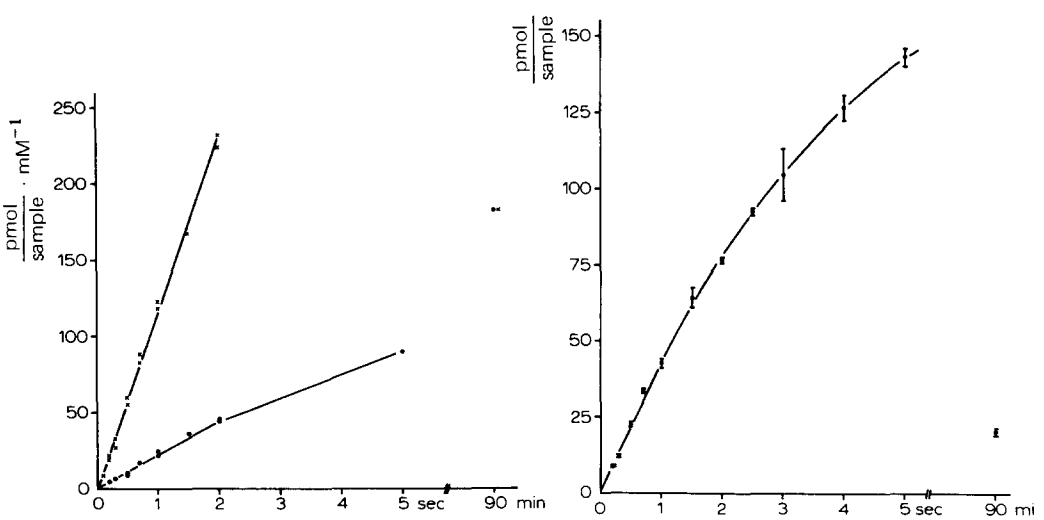

Fig. 2. Uptake of D-glucose into brush border vesicles as a function of time. The vesicles were suspended in 100 mM mannitol, 10 mM HEPES/Tris, pH 7.5. To start the incubation, D-glucose and NaCl (A) or D-glucose and NaSCN (B) were added to give the following final concentrations: (A) X———X, 10 μM D-glucose and 100 mM NaCl; ●———●, 1 mM D-glucose and 100 mM NaCl. (B) ●———●, 100 μM D-glucose and 100 mM NaSCN. The Na^+ gradient applied together with the substrate leads to a transient accumulation of D-glucose. The values at 90 min show the level of D-glucose when complete equilibration is reached. All the incubations were run at room temperature. Each sample contained about 150 μg protein.

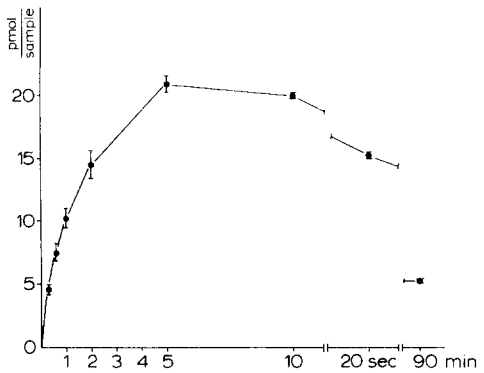

Fig. 3. Uptake of L-methionine (13 μM) into brush border vesicles in the presence of an initial gradient of NaSCN (100 mM outside—0 inside). Means and standard deviations from 2—5 determinations are shown. Each sample contained about 250 μg protein.

permeance of the anions present in the incubation medium. Fig. 2 shows the time course of D-glucose uptake in the presence of an initial gradient of either 100 mM NaCl (Fig. 2A) or 100 mM NaSCN (Fig. 2B) (100 mM outside—0 inside; between the two anions, SCN^- is the more permeant one). In all cases, D-glucose uptake was found to be practically linear during the first 2 s and to show an ever increasing deviation from this initial uptake rate thereafter. The data obtained also indicate the sensitivity and the reproducibility of the device even at incubation times as short as 0.2 s.

The linear uptake during the initial 2 s cannot be safely predicted on theoretical grounds, because transport is measured under conditions of a dissipating ion gradient. In addition, the diffusion potential generated by the SCN^- gradient might undergo drastic changes. The observed linearity indicates that the system, nevertheless, is in a steady state during these 2 s. This finding might be explained by the following arguments: (a) Measurements of ^{22}Na uptake showed that by 2 s the concentration inside the vesicles was 7 mM or less. Experiments not presented here indicated that the *trans*-inhibition of internal sodium on the influx of D-glucose became substantial only at a sodium concentration inside above 5—10 mM. (b) If the permeancy of sodium is not negligibly small compared to that of SCN^-, the initially established diffusion potential $\Delta\psi$ depends on the ratio of the permeabilities of these two ions (see Goldman's equation [11]). This potential should remain constant until the SCN^- concentration inside the vesicles is sufficient to cause the SCN^- distribution ratio to become the dominating factor in determining $\Delta\psi$. (c) It may be deduced from equations derived by Geck and Heinz [12], that at high values of transmembrane potentials the substrate flux may be invariant to changes in $\Delta\psi$.

In contrast to the behaviour of D-glucose, L-methionine uptake was non-linear even during the first 0.6 s, measured under identical conditions as D-glucose (Fig. 3). In addition, L-methionine accumulation is maximal at a much earlier time than D-glucose, at about 5 s. Conventional measurements at 15 s and later can only record the falling slope of this curve and are therefore of much lower sensitivity. The difference in the behaviour of D-glucose and L-

methionine may reflect a different responsiveness of their respective transport systems towards Na^+ concentrations and potentials, a finding already noted in measurements in the intact intestine [13].

Determination of kinetic parameters of D-glucose transport

D-Glucose uptake was measured at various concentrations in order to determine K_m and V under different experimental conditions. In the experiments where an initial sodium gradient was applied, 2-s incubations were chosen because these values still represent initial uptake rates at all concentrations tested. The uptake rates, when plotted according to Eadie and Hofstee (Fig. 4)

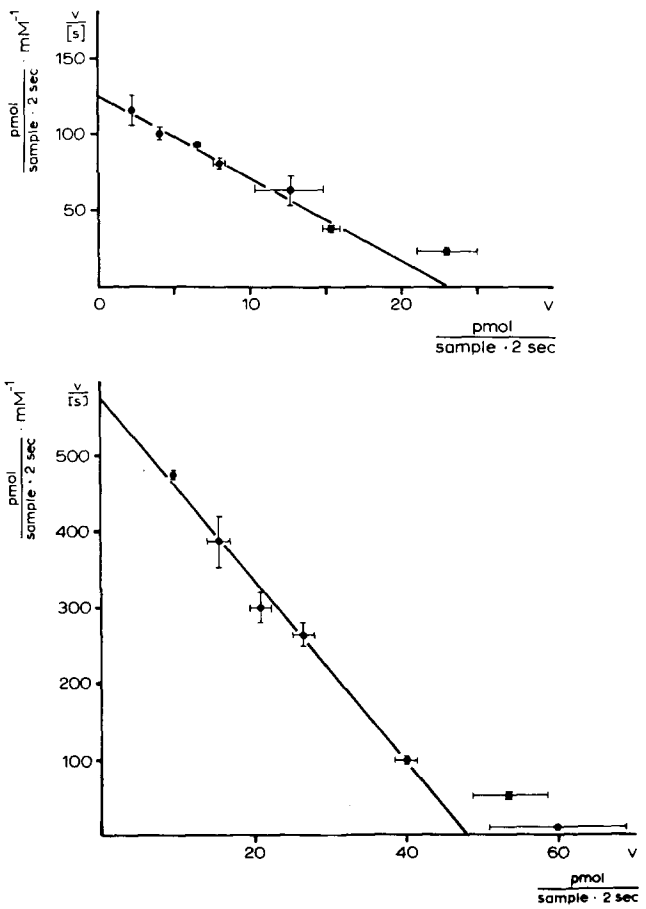

Fig. 4. Saturation of Na^+-dependent D-glucose transport. The 2 s uptake of D-glucose was measured at different D-glucose concentrations and plotted according to Eadie and Hofstee. The experimental conditions correspond to those of Figs. 2A and 2B. The data of A were measured in the presence of an initial gradient of 100 mM NaCl and the following D-glucose concentrations: 20, 40, 70, 100, 200, 400 and 1000 μM. The data of B were determined in the presence of an initial gradient of 100 mM NaSCN and the following D-glucose concentrations: 20, 40, 70, 100, 400, 1000 and 5000 μM. The position of the lines were calculated by linear regression through all points except those corresponding to the concentrations of 1 and 5 mM D-glucose. The values are means and standard deviations of three measurements. Each sample contained about 150 μg protein. The kinetic constants derived from these graphs are collected in Table I.

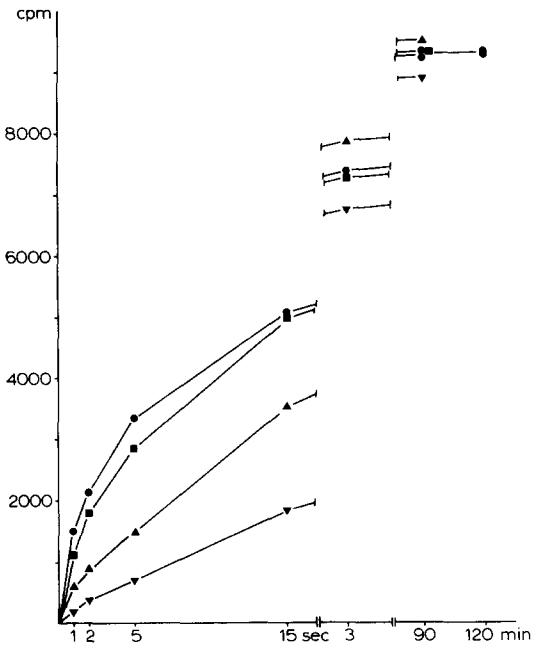

Fig. 5. Time course of D-glucose uptake in a tracer exchange experiment (zero net flux). Brush border vesicles were pre-equilibrated for 90 min with 100 mM NaCl and one of the following concentrations of unlabelled D-glucose: ●———●, 20 μM; ■———■, 1 mM; ▲———▲, 5 mM; ▼———▼, 50 mM. The incubation was started by adding an equal amount of D-[^{3}H]glucose to the samples, so that all incubations had the same amount of tracer, but different specific activities. The values shown are means of 2—4 determinations. The level reached after 90 min represents the amount of tracer inside the vesicles when equilibrium has been reached. The fact that the tracer reaches the same equilibrium level at all the different D-glucose concentrations proves that the saturation found at the initial phase of the incubation cannot be due to a displacement from D-glucose binding sites.

give straight lines for a wide range of substrate concentrations and therefore permit a precise determination of the kinetic parameters K_m and V.

In addition, tracer exchange experiments were performed to measure the flux of D-glucose at conditions of zero net uptake, i.e. when sodium, anions and D-glucose are present at the same concentration on both sides of the membrane. In the experiments shown in Fig. 5, the brush border vesicles were equilibrated with 100 mM NaCl and various concentrations of unlabelled D-glucose. Labelled D-glucose was then added in tracer concentrations to start the incubation. For the determination of the K_m, values measured at 1 s were considered to be the most reliable figures, although in this case they may only be approximations of the true initial rates (Fig. 6).

The macroscopic kinetic parameters appear to be strongly dependent upon the experimental conditions (see Table I). This is not surprising in view of their complex kinetic significance. In the equations derived by Goldner et al. [14] for D-glucose transport by rabbit intestine, as well as in the more generalised equations presented by Geck and Heinz [12], K_m and V appear as complex functions dependent upon internal and external sodium concentrations, association constants of the substrates, and the size and ratio of the transloca-

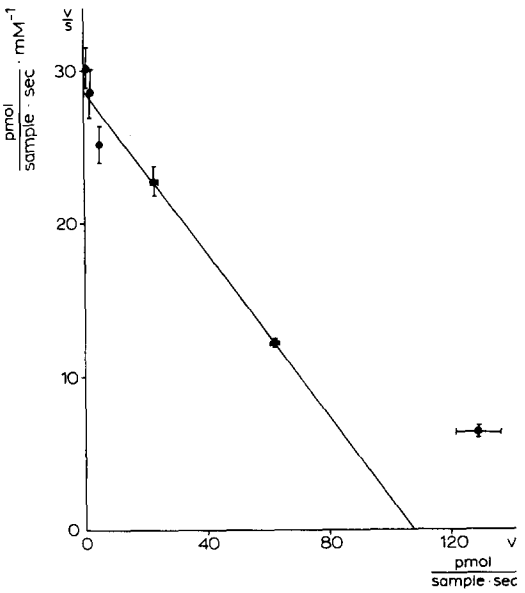

Fig. 6. Saturation of D-glucose flux in a tracer exchange (zero net flux) experiment. D-Glucose taken up at an incubation time of 1 s was plotted according to Eadie and Hofstee. The values are means and standard errors of three determinations. The D-glucose concentrations were: 20 μM, 50 μM, 200 μM, 1 mM, 5 mM, 20 mM. The concentration of NaCl was 100 mM on both sides of the membrane. The linear regression line was calculated from the five lowest D-glucose concentrations. Omitting the value at 20 mM D-glucose concentration is justified, because diffusion may become a substantial component of 'uptake' at such high substrate concentrations, therefore showing an increasing deviation from linearity. The K_m determined from the slope of the line is 3.7 ± 0.3 mM. Each sample contained about 150 μg protein.

tion rates of the loaded and unloaded carrier. In addition, as a consequence of the rheogenic character of the system, at least one of these translocation rates must be modulated by transmembrane potentials; the changes observed when NaCl was substituted for NaSCN under gradient conditions might be explained in this way. Our preliminary results are too few to allow the choice of a proper model for the observed behaviour of D-glucose transport. However, they show that short-time incubation experiments can yield reliable figures for both the half-saturating concentration and the maximal flux, and that detailed kinetic studies can be performed with brush border membrane vesicles. On the other hand, one has to be aware of the limitations of such kinetic studies, the most

TABLE I

COMPARISON OF THE K_m AND THE V

K_m and V were determined under the different experimental conditions described in this publication.

Type of experiment	K_m (mM)	V (pmol/mg protein per s)
NaSCN gradient (Fig. 4B)	0.080	150—200
NaCl gradient (Fig. 4A)	0.180	70—80
Tracer exchange (Fig. 6) (zero net flux)	3.7	500—650

important being that at present not all of the experimental conditions under which these experiments are performed can be specified. We cannot determine the exact size of either the transmembrane potential or the transient pH changes occurring in the presence of a sodium gradient due to the Na^+/H^+ exchange system described in these membranes [15]. In spite of these limitations, we believe that the possibilities to characterise the brush border transport systems by their kinetic behaviour have not yet been exhausted.

The validity of the kinetic parameters determined under the conditions of a sodium gradient (as shown in Fig. 4) might be questioned. When the concentration of D-glucose is increased, not only the D-glucose flux is increased, but also the D-glucose-coupled uptake of Na^+ via the D-glucose-Na^+ cotransport system. This additional Na^+ flux might induce a reduction of the diffusion potential, the reduction being more pronounced with increasing D-glucose concentrations and ultimately leading to a complete dissipation of the driving force for D-glucose transport. If this objection holds true, D-glucose uptake values at different D-glucose concentrations cannot be compared to each other, because they are determined under different transmembrane potentials. The V merely represents the flux of D-glucose at which the driving force is completely abolished, and the K_m indicates the D-glucose concentration at which half of this limiting flux is reached. Therefore, neither of these two parameters actually describes a property of the transport protein itself; they only reflect physical and geometrical properties of the vesicle membrane.

We cannot disprove this argument at present, but we want to present some evidence against it:

(a) During the initial 2 s, the amount of sodium entering the vesicles together with D-glucose (at the concentration of 100 μM outside, i.e. at its K_m) is less than 10% of the total sodium influx, when a gradient of 100 mM NaSCN is applied. Attempts to measure the increase in sodium influx due to the presence of D-glucose were not successful under these conditions. (However, Na^+ influx was increased to a measurable extent by the simultaneous influx of D-glucose, when the concentration of D-glucose was 1 mM and that of NaSCN was 10 mM; see ref. 6).

(b) If the maximal D-glucose flux is determined solely by the dissipation of the transmembrane potential (due to the Na^+ carried along with D-glucose), the maximal flux should remain constant, even when the number of transport proteins is reduced within certain limits, because the size of the additional sodium flux (= D-glucose flux) required for dissipating the membrane potential is primarily determined by the total permeability of the membrane for the anions and the cations and not by the number of D-glucose-transporting proteins. However, since a reduced number of transport proteins means a reduced flux of D-glucose at each concentration of the substrate, a higher D-glucose concentration is then required to reach the maximally attainable flux. If this argument holds true, an increase in the K_m should be observed without a change in the V. An experimental situation which can be considered to involve a reduction in the number of transporting sites was found in our studies on the *trans*-inhibition of D-glucose influx by internal sodium. Influx of D-glucose was measured, in the absence of internal D-glucose, at a constant inward gradient of 100 mM SCN^- and a constant outer concentration of 100 mM Na^+, but varying

concentrations of Na^+ initially present inside the vesicles (as Na_2SO_4). If this situation is interpreted within the model suggested by Goldner et al. [14], a fraction of the transport proteins should be in the non-productive state of the sodium · protein complex facing the interior of the vesicles and should withdraw an increasing number of transport proteins from their transport function as the internal Na^+ concentration is increased. This withdrawal should kinetically appear as an inhibition which is not competitive. Experiments have indeed shown that the presence of 50 mM Na^+ inside the vesicles reduces the V by about 2/3 without changing the K_m (data not shown). Whatever the exact reason for this reduction in the V, this finding can hardly be reconciled with the predictions made above. The lack of variation in the K_m in jejunum and ileum, in spite of a 3-fold difference in the V points to the same conclusion.

(c) The inhibition of phlorizin binding by D-glucose, measured at an incubation time of 2 s in the presence of a gradient of 100 mM NaSCN (for details see ref. 16), gave a K_i of the order of 100—200 μM, in good agreement with the K_m (data not shown). This inhibition of phlorizin binding cannot be attributed to a change in the potential, because L-methionine did not inhibit phlorizin binding at any concentration up to 5 mM. The transport rates of D-glucose and L-methionine were about the same in this experiment, indicating that Na^+ taken along by the non-electrolytes must have been of the same order of magnitude.

It seems very likely, therefore, that the kinetic parameters described in this text can be fully ascribed to the behaviour of the transport agency itself.

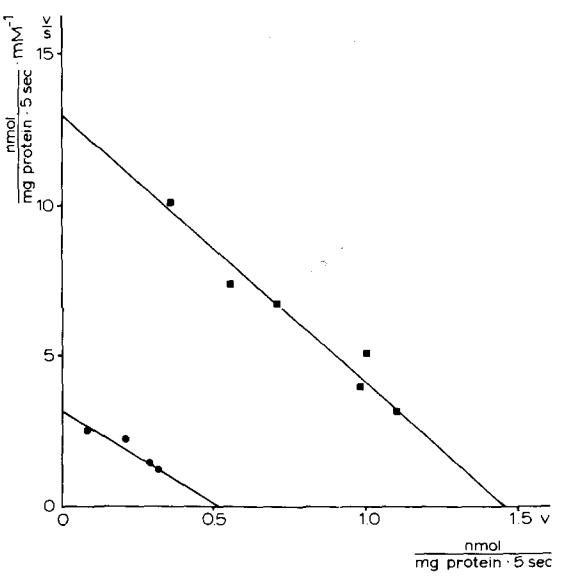

Fig. 7. Eadie-Hofstee plot of D-glucose uptake in the presence of an initial gradient of 100 mM NaSCN into brush border vesicles prepared from rabbit jejunum (■———■) and from ileum (●———●). The D-glucose concentration was varied from 30 to 356 μM. The incubation time was 5 s. Each value is a mean from 10 determinations measured on brush border vesicles from 2—3 preparations.

TABLE II

D-GLUCOSE TRANSPORT IN RABBIT INTESTINE

Kinetic constants

	K_m	V
Jejunum	185 ± 73 μM	1.45 ± 0.02 *
Ileum	145 ± 17 μM	0.45 ± 0.07 *
Significance	$P > 0.6$	$P \ll 0.01$

* nmol/mg protein per 5 s.

TABLE III

SUCRASE SPECIFIC ACTIVITY

	Jejunum	Ileum	Significance
Homogenate	0.23 ± 0.02 *	0.16 ± 0.01 *	$P < 0.05$
Vesicles	1.70 ± 0.14 *	1.04 ± 0.06 *	$P < 0.05$
Purification	7.7 ± 1.1	6.7 ± 0.6	

* μmol/min per mg protein.

Comparison of the kinetic parameters of D-glucose transport in jejunum and ileum

Measurements were made of D-glucose transport by brush border vesicles prepared from rabbit jejunum and ileum. Since the first experiments were performed manually at 5 s, the automated experiments were done at the same time. Since our measurements on vesicles from the total intestine gave comparable kinetic parameters at 2-s and at 7-s incubations, the data obtained at 5 s can be taken as a reasonably good measure of the initial rates of D-glucose uptake. The data in Fig. 7 and Table II show that the K_m is not significantly different in rabbit jejunum and ileum. However, the maximal flux in jejunum is more than three times that in the ileum. Using sucrase as a brush border marker, the data in Table III indicate that although there is more sucrase present in the jejunum than in the ileum, the relative purification of this enzyme in the two types of vesicle preparations is not different. Therefore, the higher glucose maximal flux in the jejunal vesicles is not due to the jejunal vesicles being purer than the ileal vesicles. The data presented here are in good agreement with those determined for the rat by Hopfer et al. [17], who found the half equilibration time $t_{1/2}$ to be about three times greater for the ileum than for the jejunum.

Acknowledgements

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