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SUGAR UPTAKE INTO BRUSH BORDER VESICLES FROM DOG KIDNEY

II. KINETICS

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Summary

The kinetics of D-glucose transport over the concentration range 0.07—20 mM have been investigated in a vesiculated membrane preparation from dog kidney cortex.

1. A sodium-dependent and a sodium-independent component of D-glucose uptake are observed. The sodium-dependent component is phlorizin sensitive ($K_I \approx 0.6$ μM) and electrogenic.

2. The sodium-dependent component of D-glucose uptake yields non-linear Eadie-Hofstee plots consistent with the presence of high (G_H) and low (G_L) affinity sites ($K_H \approx 0.2$ mM, $K_L \approx 4.5$ mM, $V_L/V_H \approx 7$ at pH 7.4, 25°C, 100 mM NaCl gradient). Alternative explanations are cooperative effects or non-Michaelis-Menten kinetics.

3. The initial uptake of D-glucose increases as the intravesicular membrane potential become more negative but the numerical values of K_H and K_L show little, if any, change.

4. α-Methyl-D-glucoside transport is also sodium dependent and phlorizin sensitive ($K_I \approx 1.9$ μM).

5. In contrast to the results for D-glucose, the sodium-dependent component of α-methyl-D-glucoside uptake exhibits a nearly linear Eadie-Hofstee plot consistent with a single carrier site with $K_m \approx 1.9$ mM and $V_{max} \approx 27$ nmol/min per mg protein at pH 7.4, 25°C, 100 mM NaCl gradient.

6. The kinetics of D-glucose transport in newborn dog kidney are similar to those in the adult except that the low affinity (G_L) system appears to be less well developed.

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

Introduction

Detailed knowledge about substrate-transporter interactions in intact epithelial membranes is essential for a better understanding of the molecular process of transport as well as a necessary prerequisite for monitoring solubilization, isolation and eventual reconstitution studies. In the previous paper of this series [1] we have described an in vitro membrane vesicle preparation from dog kidney cortex. We have demonstrated that this vesicle system contains a sodium-dependent, phlorizin-sensitive D-glucose transporter with similar specificity characteristics to those seen in vivo [2] in the brush border membrane of the proximal tubule. In this paper we extend our previous work to an investigation of the kinetics of this D-glucose transporter.

One of the major results of the present study concerns several new observations about the luminal glucose transport system (G transporter). When the sodium-dependent component of the initial velocity of D-glucose uptake is varied as a function of extravesicular substrate concentration the Eadie-Hofstee plot is always curvilinear. This finding is inconsistent with the behavior of a single carrier obeying Michaelis-Menten kinetics. We have virtually ruled out the possibility that metabolism, bacterial contamination or other organelle contamination are responsible for this behavior. D-Glucose and α-methyl-D-glucoside are known to share the G transporter in vivo with approximately the same affinity [3]. However, under the same experimental conditions as employed for D-glucose but using α-methyl-D-glucoside as a test substrate, we consistently obtain kinetic data more readily compatible with the existence of a single transporter with K_m = 1.9 mM. Despite this difference in their kinetic behavior, D-glucose and α-methyl-D-glucoside exhibit similar sodium dependence and phlorizin sensitivity in our vesicle system as well as being highly mutually competitive.

Methods and Materials

A. Kinetic studies. Vesiculated membrane fragments were prepared from dog kidney cortex as previously described [1]. On average, measured relative to the initial cortex homogenate, the "final vesicle fraction" used in our experiments showed a 10-fold enrichment in alkaline phosphatase activity (the brush border membrane marker), a 4-fold enrichment in Na^+ and K^+-dependent ATPase activity (the antiluminal membrane marker) and a 4-fold reduction in succinic dehydrogenase activity (the mitochondrial marker).

The "final vesicle fraction" was suspended in Buffer A (1 mM Tris-HEPES * containing 100 mM D-mannitol) for "timed uptake studies" and in Buffer A/300 (1 mM Tris-HEPES containing 300 mM D-mannitol) for "initial uptake studies". The experimental procedures were identical to those given in ref. 1 and 4. Briefly, at time zero 200 μl of incubation medium made up of Buffer A containing L-[^{3}H]glucose, the ^{14}C-labeled sugar under investigation and other additions as noted was added to 50 μl of the "final vesicle fraction". Aliquots

* 1 mM Tris-HEPES: 1 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) buffered with Tris to pH 7.4.

were taken at appropriate times and diluted in ice-cold "stop solution" then rapidly filtered through a Millipore filter (HAWP 0.45 μm). The filters, which retained the vesiculated membrane fragments, were dissolved in Bray's solution and counted.

The initial velocity of uptake was measured after 15 s of incubation at 25°C. In all "initial uptake studies" for D-glucose and α-methyl-D-glucoside the simultaneous uptake of L-glucose at the same concentration has been subtracted from that of the sugar under investigation to correct for non-stereospecific effects such as binding or trapping by the membranes and filters [1] and a small leak due to simple diffusion. The result is referred to as the stereospecific component of uptake. (The initial uptake of L-glucose was found to vary linearly with concentration in the range 0.07 to 20 mM. Therefore, in most experiments, the L-glucose uptake at a given concentration was calculated from the observed uptake of 1 mM L-glucose.)

All experiments were carried out in triplicate. The errors indicated are the S.E. values.

B. Materials. All materials used were of the highest chemical grade available. Unlabeled L-glucose, D-glucose and α-methyl-D-glucoside were purchased from Sigma Chemical Co. (St. Louis, Mo.). HEPES, Tris and phlorizin were also from Sigma. D-Mannitol was a product of the Fisher Chemical Co. (Montreal, Quebec). All radioactively-labeled materials were purchased from New England Nuclear Corp. (Boston, Mass.), and were of the highest specific activity available.

Results

A. Linearity of uptake. In this paper we are approximating the initial uptake of glucose by the uptake measured after 15 s of incubation. Since the intravesicular glucose concentration rises rather rapidly in this type of experiment (see for example Fig. 9) it is important to determine the extent to which the uptake curve deviates from linearity in this time interval. Fig. 1 shows the results of an experiment in which the stereospecific "initial uptake" of D-glucose was measured at various times at the extremes and middle of the concentration range employed in this study (0.07—20 mM). The uptake observed in the presence of 100 mM NaCl and KCl gradients have been plotted separately and the data have been normalized in order to demonstrate that within our experimental error the shapes of the uptake curves are the same for all concentrations tested. When the normalized uptakes were averaged at each time and fitted by the method of least squares to a function of the form $V_o(1 - e^{-\lambda t})$ the resulting theoretical parameters for Figs. 1a and 1b were identical to within 5% (see Figure caption). (Note that the function $V_o(1 - e^{-\lambda t})$ has been used here for illustrative purposes only. Although it seems to do an adequate job for the first 30 s of uptake it is obvious from the shape of the timed uptake curves in Fig. 9 that it is not the appropriate function to fit the data from time zero to 20 min.)

From Fig. 1 we conclude that by taking a 15 s point we systematically underestimate the initial uptake by approx. 30%. Since this error is the same for all glucose concentrations employed here in the presence of both NaCl and

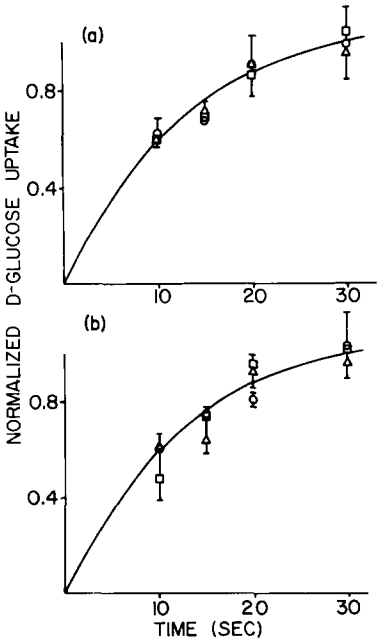

Fig. 1. The stereospecific component of D-glucose uptake is plotted as a function of time (a) in the presence of an initial 100 mM NaCl gradient (extravesicular > intravesicular) and (b) in the presence of an initial 100 mM KCl gradient. Uptake was measured at 0.06 (□), 1.0 (△) and 20 mM (○) D-glucose according to the protocol of the initial uptake studies. The incubation medium was Buffer A containing sufficient glucose and NaCl or KCl to give the final concentration indicated. The data have been normalized to show that the shape of the uptake curves is the same in each case. The solid curve drawn through the points was found by the method of least squares and is given by $V_0(1 - e^{-\lambda t})$ where $V_0 = 1.13$ and $\lambda = -0.074$ s^{-1}.

KCl gradients it would simply result in a shift in the axes of all our initial uptake figures. For this reason we have chosen to omit this correction and to plot the actual uptake observed at 15 s.

B. Kinetics of D-glucose transport. The stereospecific component of the initial uptake of D-glucose as a function of concentration is shown in Fig. 2 in the form of an Eadie-Hofstee plot. The upper three curves represent D-glucose uptake in the presence of an initial 100 mM sodium chloride gradient (extravesicular > intravesicular) with (i) no phlorizin, (ii) 1 μM phlorizin and (iii) 10 μM phlorizin in the extravesicular medium. The bottom curve shows D-glucose uptake in the presence of a 100 mM potassium chloride gradient replacing sodium chloride.

The sodium independent uptake (bottom curve) seems to represent a single transporter site with $K_m \approx 55$ mM and $V_{max} \approx 120$ nmol/min per mg protein. The characteristics of the sodium dependent uptake can be found by subtracting the sodium independent uptake at each concentration from that found in the presence of sodium. These results are illustrated as an Eadie-Hofstee plot in Fig. 3a and a Lineweaver-Burk plot in Fig. 3b. Fig. 3a is clearly curvilinear, indicating that the kinetics of the sodium-dependent component of D-glucose uptake cannot be described by a single equation of the Michaelis-Menten type. Such behavior could be accounted for by a single D-glucose transporter in which cooperative effects were taking place between protein subunits. An

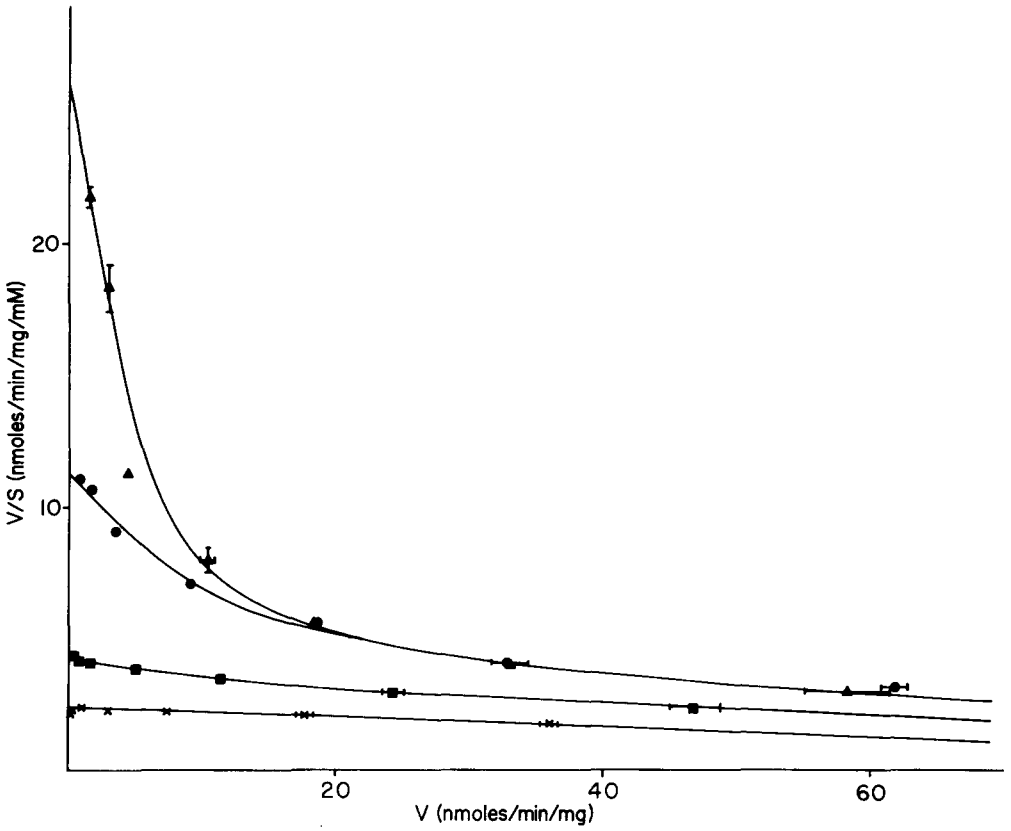

Fig. 2. An Eadie-Hofstee plot of the stereospecific component of the initial uptake of D-glucose measured as a function of glucose concentration. The points represent uptake in the presence of an initial 100 mM NaCl gradient (extravesicular > intravesicular) with no phlorizin (▲), 1 μM phlorizin (●) and 10 μM phlorizin (■) present. The bottom curve (X) shows D-glucose uptake when the NaCl gradient is replaced by a KCl gradient. The incubation medium was Buffer A containing sufficient glucose, phlorizin, NaCl and KCl to give the final concentrations indicated. The glucose concentration range is 0.07—20 mM.

alternative explanation is that there are two (or more) sodium-dependent D-glucose transporters in our preparation. The fit shown to the data in Fig. 3a assumes two such transporters and is given by the equation.

$$V = \frac{V_H \cdot S}{K_H + S} + \frac{V_L \cdot S}{K_L + S} \quad (1)$$

where V is the initial uptake of D-glucose and S is glucose concentration. The values of the parameters K_H, V_H, K_L and V_L are given in the figure caption.

There is reason to believe that the "two transporter interpretation" is the correct one although this is by no means definitely established at this time. In any case, in order to facilitate discussion of our results we will use the terms "high affinity transporter" (G_H) and "low affinity transporter" (G_L) when referring to the regions of the Eadie-Hofstee plot associated with low and high glucose concentrations respectively. The fits to the experimental data using Eqn. 1 which we have included in several of our figures have been made by eye. In order to get a more realistic estimate of the theoretical parameters we have

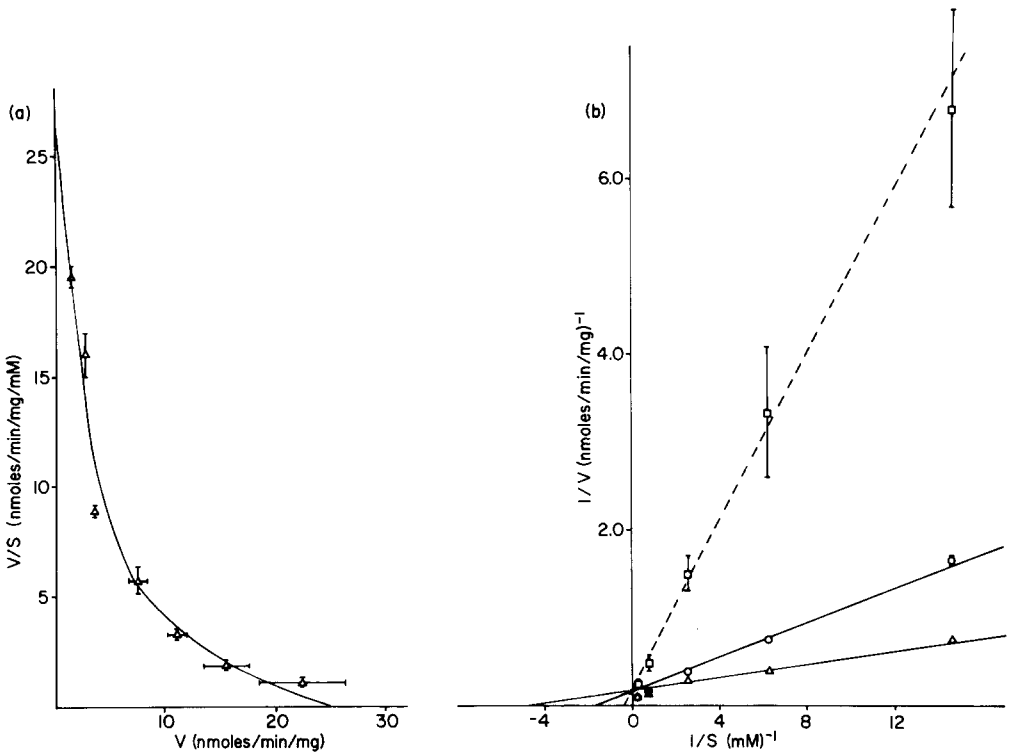

Fig. 3. The sodium-dependent component of the initial uptake of D-glucose is shown as an Eadie-Hofstee plot in (a) and as a Lineweaver-Burk plot in (b). (Note that two points at the lower end of the concentration range could not be included in the Lineweaver-Burk plot due to lack of space). These resuls were obtained from the data plotted in Fig. 2 by subtracting the sodium-independent uptake at each concentration from that found in the presence of sodium. The uptake when no phlorizin (△), 1 μM phlorizin (○) and 10 μM phlorizin (□) were present in the incubation medium are shown. The fit to the "no phlorizin" points in (a) is given by K_H = 0.16 mM V_H = 3.6 nmol/min per mg protein, K_L = 5.1 mM, V_L = 20.4 nmol/min per mg protein (see Eqn. 1 of the text).

normalized and averaged the data from eight independent experiments and fit the resulting points by the method of least squares. The results are $K_H = 0.17 \pm 0.08$ mM; $K_L = 4.5 \pm 0.6$ mM, $V_L/V_H = 6.7 \pm 2.1$ where the errors quoted are the asymptotic standard deviations.

From Fig. 3b it is obvious that G_H is competitively inhibited by phlorizin. From the graph we calculate the K_I for phlorizin to be ≈ 0.6 μM. This number is in good agreement with the value of the dissociation constant for high affinity phlorizin binding in our vesicle system ($k_d \approx 0.6$ μM at 25°C, see ref. 1). From Fig. 2 it is clear that G_L is also inhibited by low concentrations of phlorizin but the Lineweaver-Burk plot for this glucose concentration range (not shown) is difficult to interpret owing to experimental errors and uncertainties in subtracting away the effects of G_H. The data for G_L are, however, consistent with competitive inhibition by phlorizin with a K_I in the micromolar range.

Fig. 4 illustrates the inhibitory capacity of 5 mM α-methyl-D-glucoside on the sodium-dependent, stereospecific component of D-glucose uptake. It is clear that this sugar strongly inhibits both G_H and G_L at this concentration.

The above results indicate that both G_H and G_L seem to have the charac-

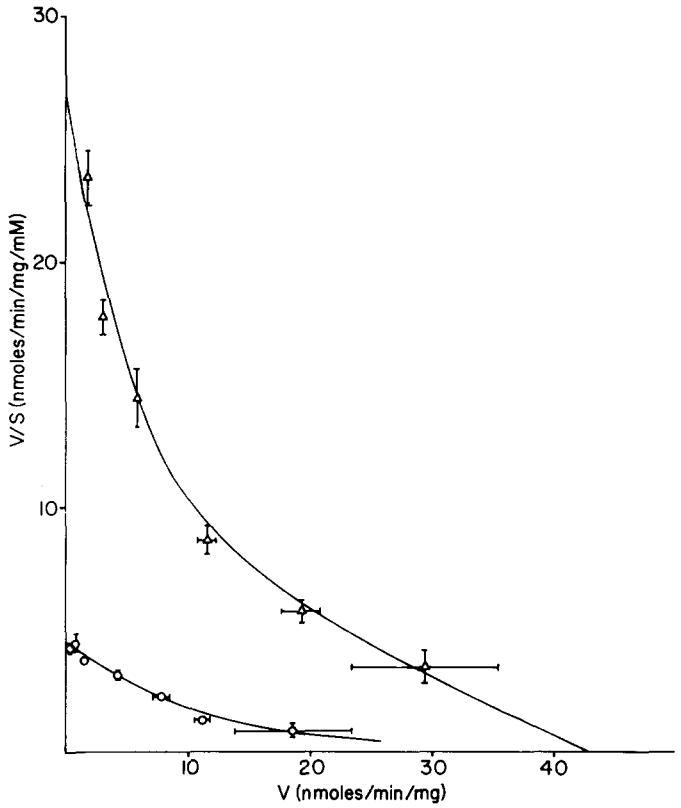

Fig. 4. An Eadie-Hofstee plot of the sodium-dependent stereospecific component of the initial velocity of uptake of D-glucose showing the inhibitory effect of 5 mM α-methyl-D-glucoside. The incubation medium for the upper curve (△) was Buffer A containing sufficient NaCl to give an initial 100 mM gradient (extravesicular > intravesicular). The result of adding 5 mM α-methyl-D-glucoside to the incubation medium is illustrated by the lower curve (○). Sodium independent uptake was subtracted as described in the text. The glucose concentration range is 0.07—20 mM. The fit to the experimental data when no α-methyl-D-glucoside was present (△) is given by K_H = 0.26 mM, V_H = 5.0 nmol/min per mg protein, K_L = 5.0 mM, V_L = 38 nmol/min per mg protein.

teristic properties usually associated with the brush border glucose transporter. They are both sodium-dependent, inhibited by phlorizin concentrations in the micromolar range and also inhibited by millimolar concentrations of α-methyl-D-glucoside, a sugar known from in vivo studies to share the G transporter with approximately the same affinity as D-glucose [3]. Furthermore, since we have previously demonstrated that D-glucose transport at the antiliminal membrane is sodium independent [1] it is not possible to associate either G_H or G_L with an antiluminal contaminant in our preparation. These correlations lead us to conclude that both G_H and G_L are localized to the luminal surface of the proximal tubule cell.

C. The kinetics of α-methyl-D-glucoside transport. Fig. 5 is an Eadie-Hofstee plot of the stereospecific component of the initial uptake of α-methyl-D-glucoside over the same concentration range and drawn to the same scale as the corresponding diagram for D-glucose (Fig. 2). Several differences in the uptakes of these two sugars are immediately obvious. First, the sodium-independent up-

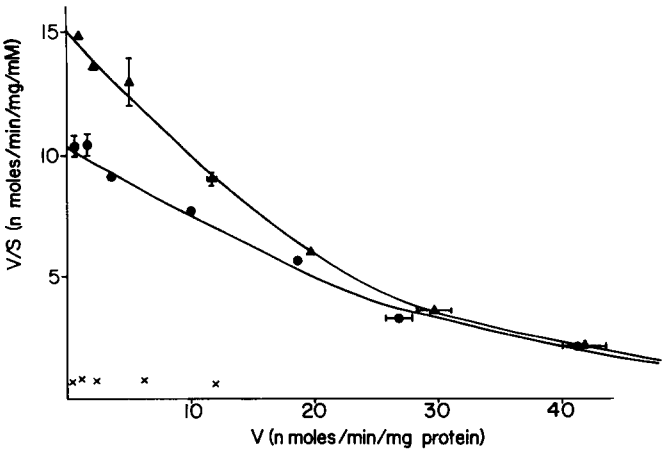

Fig 5. An Eadie-Hofstee plot of the stereospecific component of the initial uptake of α-methyl-D-glucoside measured as a function of sugar concentration. Uptake was measured with an initial 100 mM NaCl gradient in the absence (▲) and presence (●) of 1 μM phlorizin. The sodium-independent uptake (X) found when KCl replaces NaCl is also shown. Incubation media were as described for Fig. 1. The α-methyl-D-glucoside concentration range is 0.07—20 mM.

take of α-methyl-D-glucoside is markedly less than that of D-glucose. This reflects the fact that α-methyl-D-glucoside is not found to be transported by the antiluminal surface of the proximal tubule cell in vivo [5]. Thus, its only access to any contaminating antiluminal vesicles in our preparation is via simple diffusion. The fact that we see a small sodium-independent uptake of α-methyl-D-glucoside in our system could indicate the existence of an (almost insignificant) sodium-independent transporter at the brush border, but it is more likely a reflection of the fact that L-glucose does not provide a perfect measure of non-stereospecific effects for α-methyl-D-glucoside.

The second major difference between D-glucose and α-methyl-D-glucoside is best illustrated by comparing the sodium-dependent component of uptake in Fig. 3a and b, with those shown in Fig. 6. The Eadie-Hofstee plot in Fig. 6a is typical of our results for α-methyl-D-glucoside. Inspection reveals that the data points very nearly conform to a simple linear fit suggesting that there is only one significant sodium-dependent α-methyl-D-glucoside transporter in our vesicle system. Admittedly, a small low affinity component of α-methyl-D-glucoside uptake also seems to be present but this component is consistently at the borderline of significance in our studies in contrast to the D-glucose data which are always strikingly curvilinear. At this time, therefore, we do not feel justified in using the same "two-site" analysis for the two sugars. The K_m of the dominant α-methyl-D-glucoside transporter $\approx$ 1.9 mM with $V_{max} \approx$ 27 nmol/min per mg protein. The Linewater-Burk plot in Fig. 6b illustrates that this transporter is competitively inhibited by phlorizin with $K_I \approx 1.9$ μM.

D. The sodium-dependence of D-glucose transport. In order to gain a better understanding of the kinetics of D-glucose transport we have carried out several experiments related to its sodium dependence. In Fig. 7 we have plotted the sodium-dependent stereospecific component of D-glucose uptake measured in the presence of 40 mM and 140 mM sodium chloride gradients (outside > inside). Inspection of the figure suggests that G_H is relatively inactive at 40 mM

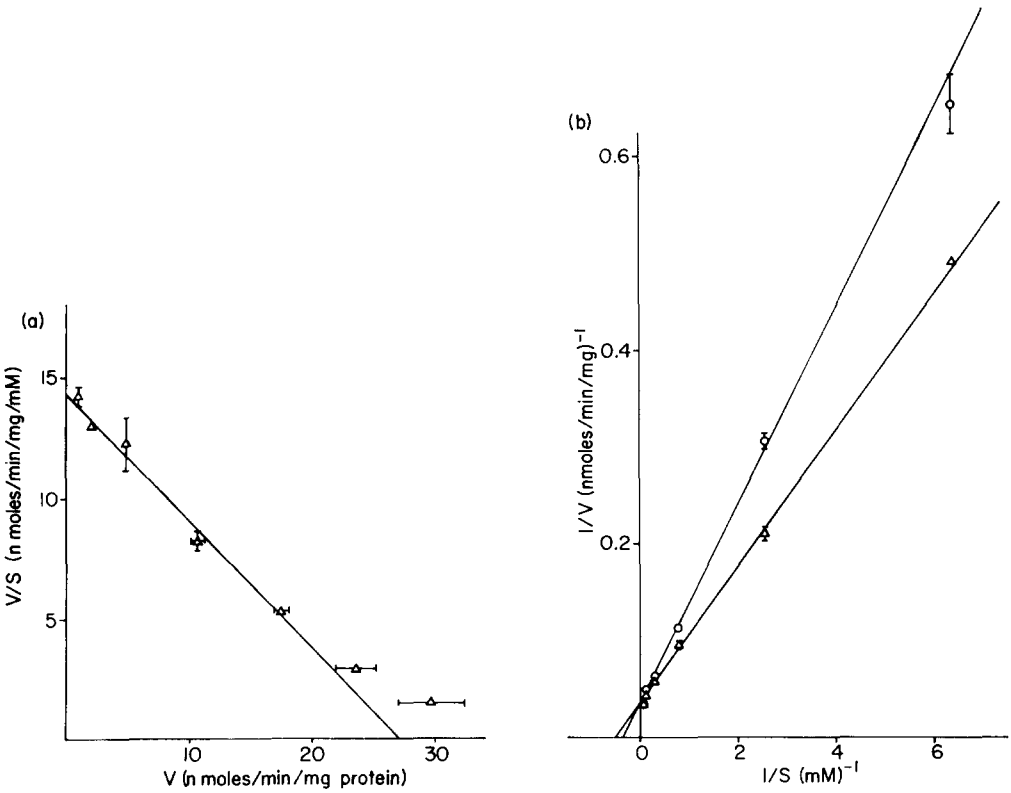

Fig 6. The sodium-dependent component of the initial uptake of α-methyl-D-glucoside is shown as an Eadie-Hofstee plot in (a) and as a Lineweaver-Burk plot in (b). The uptake when no phlorizin (△) and 1 μM phlorizin (○) were present in the incubation medium are shown. These results were obtained from the data plotted in Fig. 4 as previously described.

sodium chloride but is significantly stimulated as the concentration of this salt is increased. In contrast, G_L is hardly affected by increasing the NaCl concentration. This interpretation of the figure is substantiated by the theoretical fits to the data given in the figure caption. These show that V_H, the maximum capacity of G_H, increases over 4-fold as the NaCl concentration increases from 40 to 140 mM, while V_L remains almost unchanged.

The results of an experiment in which the initial velocity of uptake of 1 mM D-glucose was measured as a function of sodium concentration are shown in Fig. 8. The data, plotted according to Lineweaver and Burk, suggest that there are at least two sodium transporters associated with the uptake of D-glucose. One of them has a $K_m \approx 57$ mM and a $V_{max} \approx 22$ nmol/min per mg protein (assuming that one sodium ion is co-transported with each glucose molecule), while the other apparently has a considerably higher K_m. The results of this experiment correlate well with the behavior observed in Fig. 7.

E. Anion dependence. Fig. 9 illustrates the dependence of the timed uptake of D-glucose on the anion used in conjunction with Na^+ in an initial 100 mM gradient. The characteristic "overshoot" of the intravesicular D-glucose concentration above its equilibrium value is discussed in ref. 1. Replacing Cl^- by SCN^- enhances the initial uptake of D-glucose and shifts the overshoot pheno-

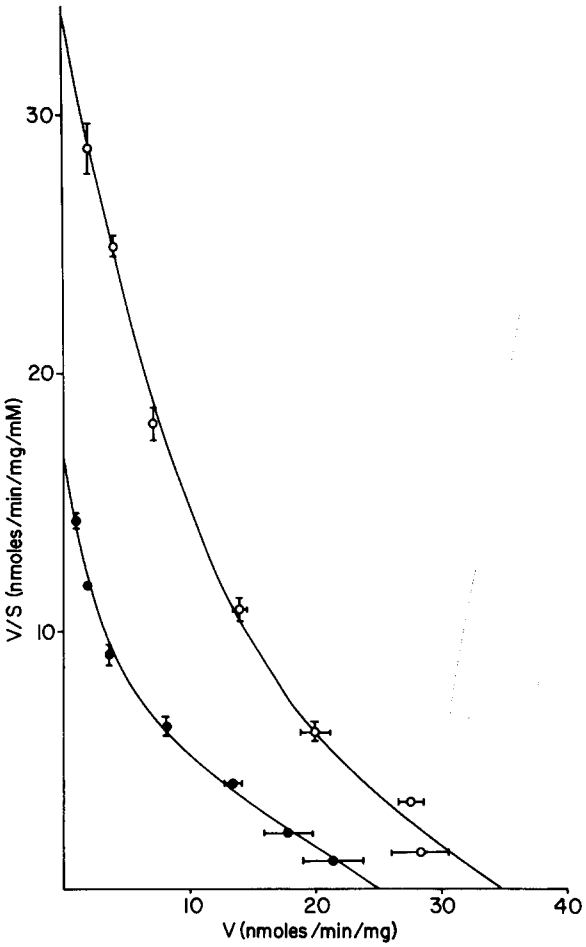

Fig. 7. An Eadie-Hofstee plot of the sodium-dependent stereospecific component of the initial uptake of D-glucose measured with initial 40 mM (•) and 140 mM (○) NaCl gradients (extravesicular > intravesicular). The glucose concentration range was 0.07—20 mM. The theoretical fits shown to the data are K_H = 0.2 mM, V_H = 2.1 nmol/min per mg protein, K_L = 3.5 mM, V_L = 23 nmol/min per mg protein for the lower curve (40 mM NaCl) and K_H = 0.35 mM, V_H = 9.7 nmol/min per mg protein, K_L = 4 mM, V_L = 25 nmol/min per mg protein for the upper curve (140 mM NaCl).

menon to earlier times, while replacing Cl^- by SO_4^{2-} reduces initial uptake and almost eliminates the overshoot. These results, which have been seen by other authors [6—8], are consistent with the hypothesis that Na^+ and D-glucose are co-transported electrogenically across the brush border [1]. When an anion such as SCN^-, for which the membrane has a larger conductivity than for Cl^- [9], is used in the Na^+ gradient a more negative intravesicular diffusion potential (membrane potential) is initially produced. This leads in turn to an enhancement of Na^+-coupled D-glucose transport, as observed in Fig. 9. SO_4^{2-}, which presumably is conducted much more poorly through the membrane than Cl^-, produces the opposite effect. The detailed shape of the curves in Fig. 9 is determined by both the conductivities and permeabilities of the ions involved.

Fig. 10 shows the sodium-dependent stereospecific component of D-glucose

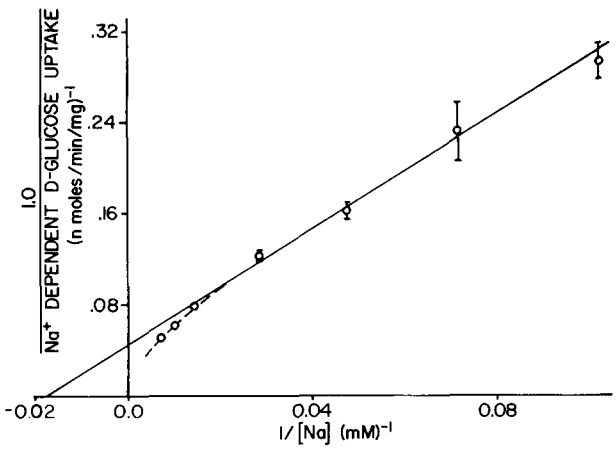

Fig. 8. A Lineweaver-Burk plot of the sodium-dependent stereospecific component of the initial uptake of 1 mM D-glucose measured as a function of Na^+ concentration. The incubation medium was Buffer A containing 1 mM D- and L-glucose and sufficient NaCl to give the initial extravesicular concentration indicated. Osmotic pressure was kept constant by replacing NaCl isoosmotically with KCl. The Na^+ concentration range shown is 10—140 mM.

uptake measured in the presence of initial 100 mM NaCl and NaSCN gradients. As before, replacing Cl^- by SCN^- presumably shifts the intravesicular membrane potential toward more negative values and enhances the uptake of D-glucose via the sodium-dependent glucose transporter. It is interesting to note, however, that the shape of the Scatchard plot as characterized by K_H, K_L and the ratio V_H/V_L (see figure caption) is not appreciably changed by the replacement of Cl^- by SCN^-. Thus, although the membrane potential can influence the net amount of substrate transported, it seems to have relatively little effect on the affinity of the transporter.

F. Metabolism, baterial contamination and other organelle contamination. Kinetic experiments for D-glucose were also carried out using highly purified brush border membranes (enrichments relative to initial homogenate were 11.7

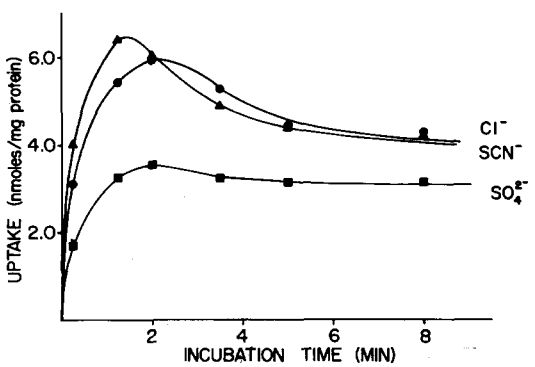

Fig. 9. The timed uptake of 1 mM D-glucose in the presence of various anions replacing Cl^-. The incubation medium was Buffer A containing sufficient NaCl (•), NaSCN (▲) and Na_2SO_4 (■) to give an initial 100 mM Na^+ gradient (extravesicular > intravesicular).

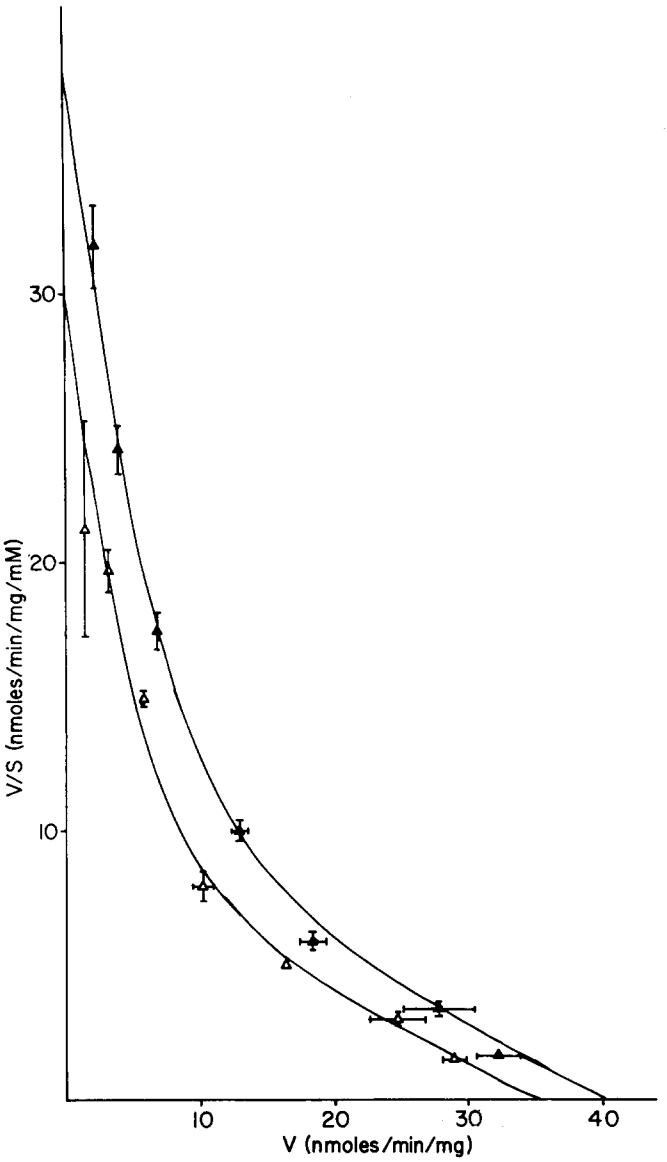

Fig. 10. An Eadie-Hofstee plot of the sodium-dependent stereospecific component of the initial uptake of D-glucose measured with initial 100 mM NaCl (△) and NaSCN (▲) gradients. The glucose concentration range was 0.07—20 mM. The theoretical fits shown to the data are K_H = 0.21 mM, V_H = 6.6 nmoles/min/mg protein, K_L = 4.8 mM, V_L = 34 nmol/min per mg protein for the upper (NaSCN) curve and K_H = 0.21 mM V_H = 5.0 nmol/min per mg protein, K_L = 4.9 mM, V_L = 31 nmol/min per mg protein for the lower (NaCl) curve.

times for alkaline phosphatase, 0.7 times for succinic dehydrogenase and 1.7 times for Na^+ and K^+-dependent ATPase) obtained from free-flow electrophoresis [10]. These membranes were not subjected to our vesicle preparation procedure but rather simply resuspended in Buffer A/300 for the experiments. The results obtained were essentially the same as those seen in Fig. 3a. Since the sodium-dependent kinetics of D-glucose uptake are invariant with these two

methods of vesicle preparation we feel that organelle contamination is an unlikely explanation for our curvilinear Eadie-Hofstee plots.

As discussed in the previous paper [1] there is no detectable metabolism of D-glucose in our vesicle system. Moreover, timed uptake experiments over 2—3 h duration show no increase in D-glucose uptake with time, as would be expected if significant bacterial contamination were present. Taken together these observations seem to rule out the possibility that metabolism, bacterial contamination or other organelle contamination contribute to the kinetic behavior we observe for D-glucose. Finally, it is worth mentioning that using maltase activity and the concanavalin A receptor as markers of the external surface of the brush border, we have found no detectable inside-out vesicle

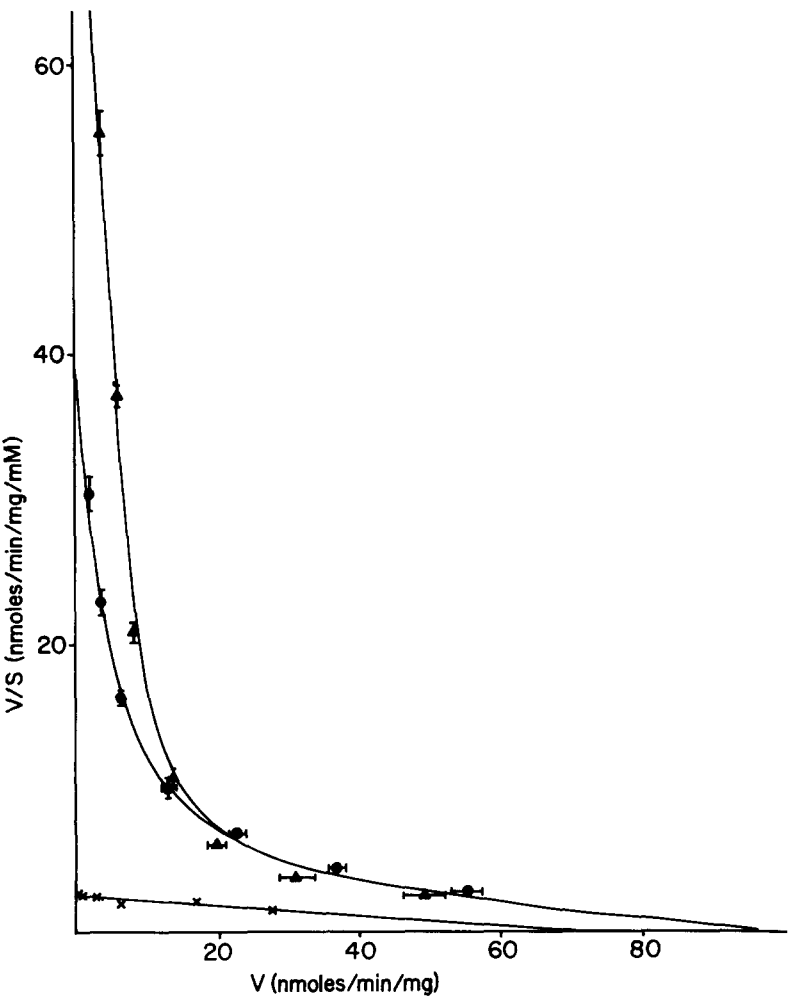

Fig. 11. An Eadie-Hofstee plot of the stereospecific component of the initial uptake of D-glucose in brush border vesicles from the newborn dog. Uptake was measured with an initial 100 mM NaCl gradient in the absence (▲) and presence (●) of 1 μM phlorizin. The sodium-independent uptake (X) found when KCl replaces NaCl is also shown. Incubation media were as described for Fig. 1. The D-glucose concentration range is 0.07—20 mM.

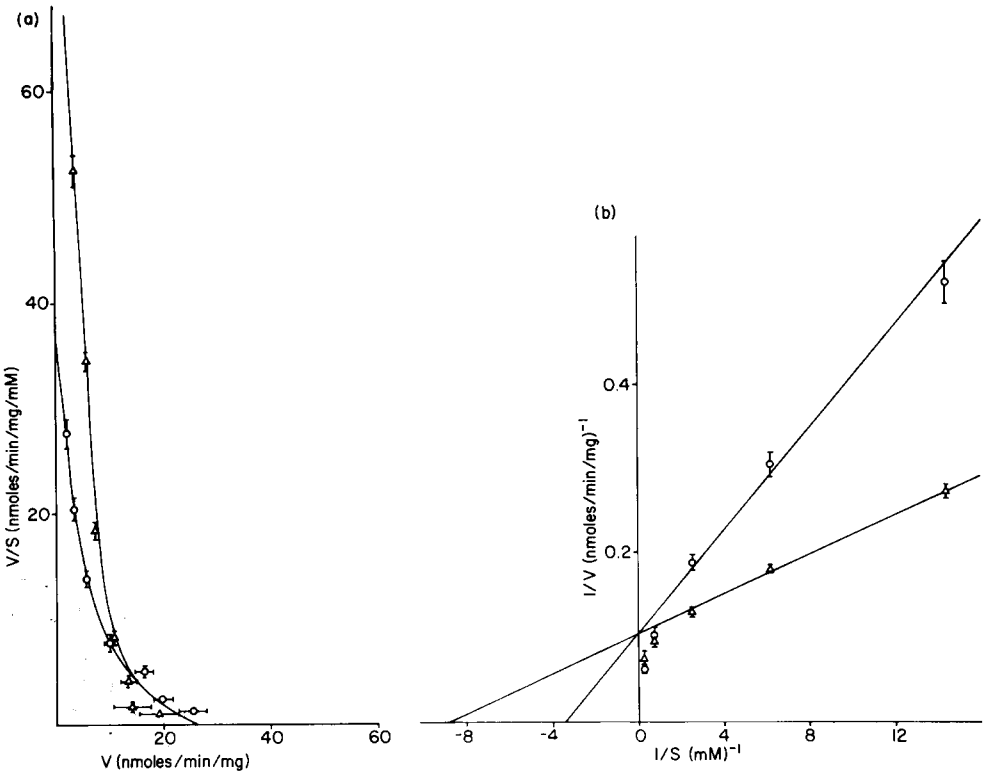

Fig. 12. The sodium-dependent component of the initial uptake of D-glucose in newborn dog brush border vesicles is shown using an Eadie-Hofstee plot in (a) and a Lineweaver-Burk plot in (b). The uptake when no phlorizin (△) and 1 μM phlorizin (○) were present in the incubation medium are shown. These results were obtained from the data plotted in Fig. 11 as previously described. The fit shown to the upper ("no phlorizin") curve in (a) is given by K_H = 0.1 mM, V_H = 8.2 nmol/min per mg protein, K_L = 6 mM, V_L = 18 nmol/min per mg protein.

population in our preparation (Silverman, M., unpublished observation).

G. Development of the G transporter. Fig. 11 illustrates the results of an experiment in which the stereospecific uptake of D-glucose was measured in a vesicle preparation from a newborn dog. The sodium-dependent component of this uptake is shown in Fig. 12. The Eadie-Hofstee plot in Fig. 12a is curvilinear as observed in the adult; however, comparison with Fig. 3a indicates that the component of uptake representing G_L in this preparation is considerably reduced in magnitude. Also G_H has a somewhat higher affinity ($K_H \approx 0.1$ mM) for D-glucose than typically seen in the adult. From the Lineweaver-Burk plot of these data shown in Fig. 12b we conclude that the inhibition of G_H by phlorizin is competitive with $K_I \approx 0.6$ μM.

Discussion

We have presented the results of a series of experiments relating to the kinetics of D-glucose transport in a vesiculated membrane fraction prepared from dog kidney cortex. The existence of both a stereospecific sodium-independent and a stereospecific sodium-dependent transport system for D-glucose

in this preparation has been demonstrated. The sodium independent system which has relatively high capacity and low affinity is probably associated with the antiluminal membrane which represents a significant contaminant in our preparation. Virtually all of the sodium-dependent uptake we observe is highly phlorizin sensitive and inhibited by millimolar concentrations of α-methyl-D-glucoside, a sugar which has been shown to interact with the brush border but not the antiluminal membrane in vivo [5]. These findings, together with our previous work on the specificity of D-glucose uptake in the vesicle system [1], lead us to associate all of the sodium-dependent uptake with the brush border surface.

The Eadie-Hofstee plot of the sodium-dependent stereospecific uptake of D-glucose is curvilinear. There are several possible explanations for this result: (i) that there are two (or more) sodium-dependent D-glucose transporters in the brush border membrane, (ii) that there is a single D-glucose transporter in the luminal membrane in which cooperative effects are taking place, or (iii) that some of the simplifying assumptions usually made in the mathematical analysis of simple transport models do not apply and Michaelis-Menten type kinetics are not applicable to this system. Our present data tend to support the hypothesis that there are (at least) two sodium-dependent D-glucose transporters in the brush border membrane since we have been able to demonstrate that the high (G_H) and low (G_L) affinity regions exhibit different characteristics. Namely, G_L has a higher affinity for sodium than G_H and is less prominent in the newborn animal. This latter fact suggests that G_L is a product of later development. Assuming the existence of two transporters and averaging over a number of experiments we have estimated that the kinetic parameters defined by Eqn. 1 are given by $K_H \approx 0.2$ mM, $K_L \approx 4.5$ mM and $V_L/V_H \approx 7$ at 100 mM NaCl and 25°C. At 1 mM D-glucose (the concentration at which the experiments in our previous report [1] were carried out) each of these terms (or "transporters") contributes roughly equally to the sodium-dependent uptake. We fully expected the kinetic behavior of D-glucose and α-methyl-D-glucoside to be nearly identical. However, on the basis of our measurements α-methyl-D-glucoside shows only one significant sodium-dependent transporter. Since this compound clearly inhibits both G_H and G_L we must tentatively conclude that it binds to both transporters but is either only transported by one of them or transported by both with almost equal affinity.

The existence of two D-glucose transporters in the kidney with the phlorizin sensitivities observed would not be inconsistent with the in vivo data from this laboratory or from other investigators. However, the functional significance of having two transporters is not clear. Data from chick embryo fibroblasts [11] suggest the presence of both high and low affinity sugar transport mechanisms. Moreover, in this cell type transcriptional events can modulate the high and low affinity carriers differentially. Thus, there may be advantages in having two glucose transporters localized to the luminal surface in mammalian epithelia. Alternatively, the presence of two transporters could reflect heterogeneity of cell type as a function of length in the proximal tubule.

Using microperfusion techniques Von Baeyer et al. [12] have estimated the K_m for D-glucose in the rat renal proximal tubule to be 10.8 mM at 37°C. Given the species and temperature differences and the possible effects of mem-

brane isolation procedures this number is in good agreement with our value for K_L. Since these authors used D-glucose concentrations in the range 2.0—30 mM their experiment does not rule out the existence of a higher affinity transporter. Aronson and Sacktor [13] have studied the kinetics of D-glucose in a brush border vesicle preparation from rabbit kidney. They estimate that the $K_m \approx 0.09$ mM at 20°C and 40 mM NaCl for the sodium-dependent component of uptake in their preparation. Busse et al. [14] have found a sodium-dependent phlorizin-sensitive transporter with $K_m \approx 0.07$ mM in a crude brush border membrane fraction. In addition they observe a sodium-independent phlorizin insensitive component of D-glucose uptake with $K_m \approx 3$ mM which they also associate with the brush border membrane [14,15]. However, the phlorizin insensitivity of this sodium-independent site suggests that the problem of antiluminal membrane contamination may not have been satisfactorily dealt with by these authors [2].

The affinity of D-glucose for its transporter at the luminal surface can also be estimated from phlorizin binding measurements. We have found the K_I of D-glucose for phorizin binding in our vesicle preparation to be ≈3 mM at 37°C [1]. This number correlates well with our estimate of K_L. Further experiments are required to clarify the relationship between D-glucose transport and phorizin binding. At the present time we have only been able to detect one high affinity phlorizin binding site in our vesicle preparation [1].

Diedrich [16] has been able to estimate the time required for a glucose molecule to pass through the brush border membrane in vivo. By measuring the maximal capacity of renal tubules to reabsorb glucose and equating the number of glucose transporters with the number of high affinity phorizin receptors he calculates a value of 0.04 s for the mean transit time. Our experimental results allow us to compute this same number independently in vitro. In the previous paper we have estimated the number of high affinity phlorizin receptor sites to be approx. 200 pmol/mg protein in the vesicle system. The maximal rate of sodium-dependent D-glucose uptake at 140 mM NaCl can be taken from Fig. 7 to be approx. 35 nmol/min per mg protein at 25°C. Experiments not shown here which were carried out at 37°C indicate that this value will be approx. 90 nmol/min per mg protein at physiologic temperatures. Thus, the mean transit time across the brush border membrane in our vesicle preparation is approx. 0.3 s at 25°C and approx. 0.1 s at 37°C. These figures are necessarily overestimates since not all of the phlorizin receptors we observe are necessarily associated with closed membrane vesicles (i.e., capable of vectorial D-glucose transport). Given the "trauma" of the membrane preparation procedure and the relatively non-physiologic nature of our buffers we feel that our values of the mean transit time agree well with the in vivo estimates.

A potential problem has been pointed out relating to the measurement of the transport of Na^+-coupled substrates in a vesicle preparation. In our experimental system the difference in the electrochemical potential for Na^+ between the inside and outside of the vesicle provides the driving force for active D-glucose uptake. This quantity is given by

$$\mu_{Na^+} = \Delta\psi - 2.3 \frac{RT}{F} \log \frac{[Na^+]_0}{[Na^+]_i}, \tag{2}$$

where $\Delta\psi$ is the membrane potential and $2.3\,RT/F$ is $\simeq$59 mV at room temperature. Since the transport of D-glucose is coupled to that of Na^+ the flux of glucose is capable, in principle, of affecting either term on the right of Eqn. 2; the first because it carries charge into the vesicle and the second because it depletes the Na^+ gradient. However, Kinne et al. [6] have measured the uptake of 100 mM NaCl into rat brush border vesicles. They find that the initial uptake of NaCl is 50 times that of 1 mM D-glucose. We have found similar results in our laboratory. In our kinetic experiments the initial sodium-dependent uptake of 20 mM D-glucose is approximately 3 times larger than that of 1 mM D-glucose. Thus, even at the largest glucose concentration used in our studies the glucose load only results in a 6% depletion of the Na^+ gradient. The effect of Na^+-coupled D-glucose entry on membrane potential is more difficult to evaluate. $\Delta\psi$ is a function of the relative conductivities for Na^+ and Cl^- through the membrane and neither of these is known in our system. Thus, it is not clear how much this quantity will be changed by the Na^+ leak provided by D-glucose. However, in view of the relatively small effect of D-glucose entry on Na^+ permeability there is no a priori reason to believe that its effect on conductivity will be large. In any event, any decrease in μ_{Na+} resulting from Na^+-coupled glucose influx would tend to preferentially decrease the measured uptake of D-glucose at a higher concentration. Thus, this effect cannot account for our curvilinear Eadie-Hofstee plots since correcting for it would tend to make our plots more curvilinear.

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